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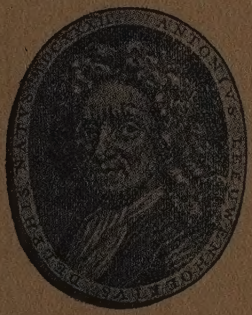

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OBSERVATIONS ON 'HEALTHY' HUMAN CARRIERS OF *PLASMODIUM VIVAX*

by

F. J. A. PAESI

(Received December 30, 1943)

With the term 'healthy' parasite carriers persons are designed harbouring parasites, which, however, do not cause them sufficient trouble to make them seek medical advice. So such persons are not actually healthy, although they are supposed to be so.

The extensive investigations of SWELLENGREBEL c.s. have made it clear that these 'healthy' carriers are of paramount significance for the epidemiology of benign tertian in this country. VAN THIEL (6) and SWELLENGREBEL and DE BUCK (4) have demonstrated i.a. that practically one subspecies of *Anopheles maculipennis* only acts as an intermediate host of *Plasmodium vivax* in our country and that the activity of the mosquito with respect to transmission is mainly confined to the second half of August and to September and October. In this period, however, the number of malaria patients (*viz.*, those, suffering from clinically recognisable accesses of fever) is small; these are met with mainly from May up till August.

So in most cases the mosquitoes will ingest the germs with which they may subsequently infect healthy persons not from diseased persons, but from 'healthy' carriers, who have suffered from malaria at an earlier date. Persons, who are infected in autumn in this way, will generally not incur their initial attack before the summer of the following year, after a period of incubation of about 9 months.

Thus another group of 'healthy' parasite carriers is formed, *viz.*, those who may expect an outbreak of the disease. This group is probably of no significance for the spread of malaria (see further on).

The important part played by the parasite carriers in the spread of benign tertian induced us in the summer 1940 to take up an investigation of the number and nature of the parasites in their blood and to collect some data on their state of health. This investigation, which has been carried out in the northern part of Amsterdam, furnished, moreover, the opportunity to judge the results of the 'autumnal quininisation'. This treatment, which was applied to all those who had suffered from one or more attacks of

fever in summer, consisted in the administration of a dose of 1 g of quinine (dose for adults) once a week, with the aim of inducing a decrease of the number of parasites in the circulating blood to such an extent that the chance of infection for mosquitoes in autumn would be markedly diminished. By such means it was hoped to stop the spreading of the prevailing epidemic, as far as Amsterdam was concerned.

For our investigation we chose a suburb in the extreme north of Amsterdam, where a great number of cases had occurred in 1939 and the beginning of 1940. This suburb was mainly populated by unemployed; these were treated by Dr. BERGHUIS, whom we are greatly indebted for his advices. In about 1200 visits thick films were made, which were then stained in the usual way by Giemsa's solution. Subsequently the number of parasites per 6000 leucocytes was counted — as is usual here — and their stage of development noted.

I CARRIERS DURING THE PERIOD OF INCUBATION

In June and July 1940 we have been looking for carriers of parasites, who might have been infected in the autumn 1939 and whose first attack was still to be expected, in families in which the number of malaria patients had been particularly high in the preceding year. We found among 174 members of 21 families 39 carriers of parasites, among whom, however, three only had not yet suffered from an attack of fever. (The others were persons, who had had their first attack already in 1937, 1938, 1939 or — as the investigation was started somewhat late in the season for malaria — shortly before, in 1940). Of those three some data are presented:

Table I

	A	B	C
Number of days which elapsed before the attack	6	5	½
Number of parasites per 6000 leucocytes	240	1	4500
Nature of the parasites	young rings fullgrown rings	young rings	fullgrown rings young rings ♂ gametocytes

C had his first attack some hours after his blood had been tested. For various reasons it was impossible to examine A's and B's blood at regular intervals up till the first attack. Among the 174 persons examined occurred, next to these three, a further e i g h t in whom n o parasites could be detected, although their first attack occurred after a lapse of 3 days(!), 2, 2, 2, 3, 4, 4 and 4 weeks respectively.

These facts seem to indicate that during the period of incubation up till a short time before the attack no parasites, or only a very small number, occur as a rule in the circulating blood. This is in agreement with the experience previously acquired in voluntarily infected persons as well as in sufferers from genuine paralysis, who had been inoculated with germs of benign tertian (SCHÜFFNER, KORTEWEG and SWELLENGREBEL (3)). It is well known that even during KORTEWEG's initial remittent no or few parasites can be traced in the blood (2).

II PARASITE CARRIERS WHO HAVE ALREADY SUFFERED FROM ACCESSES

The autumnal quininisation

Quinine was administered from August 15th till November 1st 1940. The weight of the dose had been fixed according to results arrived at by KORTEWEG (2) in Noord-Holland and by KOCH c.s. (1) in Africa and Italy. Their communications, however, bore on the administration of 1 g of quinine (dose for adults) on at least 2 succeeding days, *viz.*, every 9th and 10th, or every 6th and 7th day, the treatment being concerned with the prevention of relapses or of new infections. As the autumnal quininisation aimed at keeping parasites out of the circulating blood only, 1 g of quinine once a week was considered worth trying. More recent literature did not furnish any further encouragement.

In July and August 54 carriers of parasites were traced, who, from August 15th onward, were supposed to take the drug weekly. Among these carriers, fifteen, the blood test of whom was repeatedly positive in October, were selected for judging the effect of this particular mode of administering quinine.

As this effect was still unknown to us, we had told all of them temporarily to stop taking the weekly doses of medicine on October 1st in order to be able to trace a number of 'good' carriers of parasites. In the fifteen who were found suitable in the weeks which followed, the weekly treatment was resumed from November 1st onward (the actual quininisation was then already brought to a close).

Twelve of these fifteen had had their first attack in 1940, two of them in 1938 and one in 1939. From November 4th up till November 30st blood films were made daily, except on Sundays; on Sundays, however, they had to take the quinine.

We did not enforce the taking of the drug. An eventual success of such a compulsatory administration would not have taught us anything about the effectiveness of the method, as the actual taking of the medicine will always have to be left to the initiative of those concerned.

Nevertheless all were reminded on Saturday of their Sunday duty and each of them was asked on Monday, whether it had been fulfilled; in the few cases in which this could not be affirmed one of the following days was destined for the taking of the drug.

For a separation into two groups, one taking the medicine and the other not, the numbers obviously are too small.

Tab

Parasite counts (number of parasites per 6000 leucocytes) in

	October 1940														Nove
	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11
T. S.					48		□	—	—	1	—	—	—	□	—
A. v. D. ...							□	12	—	20	1	6	1	□	—
D. de R. ..		9300		□			□	—	—	—	—	—	—		—
W. de R. *)		60					□	1	1	—	1	—	4		18
S. F.		1					□	—	—	—	—	—	—		—
J. T.		240					□	1	5	—	3	—	1	□	15
J. Kl.		180					□	—	2	—	—		—	□	1
W. v. Z. ..		4					□		2	—	—	—	6	□	2
Adr.v.Z. ..		120					□		5	8	8	10	5	□	120
W. Reg. ..	120		□							□	120	72	120		150
T. V.				□									7	□	10
L. V.				□									720	□	720
R. v. L.	240					□							—	□	—
J. Sch.			□								2400			□	3
C. V.				□							2				—

*) Spleenless carrier.
□ Day, on which quinine was prescribed. From October 29th till November 2nd it w
— No parasites found.

The results were disappointing. Although all of the 15 carriers assured emphatically, to have taken the quinine regularly (with or without extra remindings), not one of them was wholly free from parasites during this period (see Table II).

Thirty-six times we had thus the opportunity to count the numbers of parasites in the blood 24 hours (in some cases a few hours) before as well as after the moment at which quinine was taken. After the taking of the medicine the number of parasites appeared in 14 cases smaller, in 12 cases larger and once the same when compared with the amount before the taking, whilst in one case it was negative before as well as after. In two out of 14 cases only in which the parasite count decreased, it came down to zero; in one of the latter cases the blood test remained negative for the period of observation, in the other during not more than 24 hours.

Never could any increase in the number of degenerated plasmodia be noted after the taking of the drug; gametocytes were always present.

So it is clear, that the method such as it has been followed has not led to its aim: the expelling of most of the malaria parasites from the blood circulation. It is self-evident, that the unsuccessful result may not wholly be ascribed to an insufficient dosage of quinine, as the actual taking had not been ascertained. As, however,

I

lood of 15 healthy carriers during the autumnal quininisation.

er 1940

13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
—				□	—	—	—	—	—	—	□		—	—	—		—
—	—	—	—	□	—	—	—		—	—		—	—	—	—	—	—
—	—	1	1		20	30	60	60	360	720		3000	6030	1500	150	—	3
60	—	—	—		—	—	—	—	1	5		120	—	1	300	120	120
—	—	—	—		—	—		2	—	—		20	□	1	5	—	3
10	—	—	—	□	—	—	—	—	—	—	□	—	—		—	—	—
5	8	10	60	□	10	10		10	30	6	□	5	10	10		4	1
4	5	90	90		30	540	84	180	120	120	□	2	1	—	—		—
2	2		15	□	3	4		1	1	10	□	15	10	5	15		40
150	180	48	540		720	180	210	5	1	1		—	—	—	—	1	
1	—	—	5	□	20	15	10	1	60	8	□	15	84	50	180		1
240	360	300	1200	□	360	180	180	90	300	90	□	120	480	240	25		180
—	—	—	—	□	1	—	—	—	3	5	□	1	—	—	—	4	—
	1	—	—	□	1	240	60	4	20	—		1	—	—	1	—	1
2	30	2	40		80	5	20	60	72	—		—	—	—	—	—	—

veral carriers taken on their own account.

it is quite out of the question that the medicine would never have been taken by any of the carriers and as nevertheless practically no positive results could be booked, the conclusion may be drawn, that in case this method might be taken up again — either with or without supervision of the taking of quinine — a higher dose is to be recommended.

Parasite counts in 1941

From the 15 carriers the blood film of whom had already been tested systematically during the autumnal quininisation thick films were prepared in January/February, April/May and June/July 1941 along quite similar lines: every working day during 3½ weeks at a stretch (See table III, IV and V).

It may be said here, that not one of the carriers had an actual relapse during the period of observation.

The data thus collected all confirm the already known fact, that the number of parasites in the blood of those suffering from chronic malaria varies strongly. Moreover, the variation appeared to occur with a certain regularity. In 9 out of 15 carriers we could note a regular alternation of periods in which no or hardly any parasites could be detected, with others in which their number

Table I,II — Parasite counts (number of parasites per 6000 leucocyte

	January 1941											
	20	21	22	23	24	25	26	27	28	29	30	31
T. S.	2	1	—	—	—	1		2	—	—	—	—
A. v. D.	—	—	—	—	—	—		—	—	1	—	2
D. de R.	—	—	—	—	—	—		1	2	7	14	72
W. de R. *)	90	72	420	120	60	96		300	420	120	150	120
S. F.	15	10	2	1	—	—		—	—	—	—	
J. T.	—	—	3	—	2	2		10	4	12	5	
J. Kl.	—	2	4	2	—	2		—	—	2	10	
W. v. Z.	90	48	240	180	330	25		6		—	—	—
Adr. v. Z.	4	3	1	1	—	—		1		—	—	—
W. Reg.	—	2	4	3	—	6		8	5	20	30	2
T. V.	12	5	4	2	6	5		—	—	—	1	—
L. V.	—	—	1	2	1	12		180	240	660	240	24
R. v. L.	2	—	—	—	—	—		—	—	—	—	
J. Sch.	2	1	20	40	90	90		20	30	72	48	1
C. V.		—	—	—	—	—		—	—	—	—	—

Table IV — Parasite counts (number of parasites per 6000 leucocyt

	April 1941								
	28	29	30	1	2	3	4	5	6
T. S.	—	—	—	—	—	—		—	—
A. v. D.	—	—	—	—	—	—		—	—
D. de R.	1	2	—	—	1			12	
W. de R. *)	90	30	150	180	240	150		180	10
S. F.	—	—	1	—	—	—		—	—
J. T.	—	2	1	1	—	—		—	—
J. Kl.	1	—	10	—	4	—		2	
W. v. Z.	—	—	—	—	—	—			—
Adr. v. Z.	—	—	—	—	—	—		2	—
W. Reg.	—	—	—	—	—	—		—	—
T. V.	—	—	1	—	—	—		—	
L. V.	—	—	3	12	8	12		15	1
R. v. L.	3	—	3	5	10			—	
J. Sch.	—	1	1	—	1	1		—	—
C. V.	—	—	—	—	—	—		—	—

Table V — Parasite counts (number of parasites per 6000 leucocy

	June 1941							
	9	10	11	12	13	14	15	1
T. S.	—	—	—	—	—	—		—
A. v. D.	—	—	—	—	—	—		—
D. de R.	—	—	—	—	—	—		
W. de R. *)	132	150	84	120	96	72		—
S. F.	—	—	—	—	—	—		
J. T.	15	40	6	4	—	—		
J. Kl.	3	—	—	—	—	—		
W. v. Z.	30	90	270	144	420	360		12
Adr. v. Z.	—	—	—	—	—	—		
W. Reg.	—	—	—	—	—	—		—
T. V.	—	—	—	—	—	—		
L. V.	—	—	—	—	—	—		
R. v. L.	—	—	—	1	4	—		
J. Sch.	—	—	—	—	—	—		
C. V.	—	—	—	—	—	—		

*) Spleenless carrier.
— No parasites found.

the blood of 15 healthy carriers in January and February 1941.

February 1941

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
—		—	—	—	—	—	1		—	—	—			
10		180	150	360	360	240	90		2	—	—			
80		360	600	360	60	30	1		—	—		—		
120		240	15	60	48	48	40			96		270		
—		2	—	1	—	2	10			150	300			
1		1	8	—	—	—	—		—	—	—			
6		6	8		—	5	—		4	—		—		
—		—	—	—	—	1	3		15	40	60			
—		1	7	4	4	6	7		25	10	10			
72		50	30	60	60	96	120			120	90			
—		—	—	—	—	—	1		1		1			
48		40	48	25	3	3	—				35			
7		20	15	35	60	150	25		4	1		4		
10		1	—	2	1		1				8			
—		—	—	—	—	—	—			—		—		

the blood of 15 healthy carriers in April and May 1941.

941

7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
—	—	—	—		—	—	—	—	—	—		—	—	—
—	—	—	—		—	—	—	—	—	—		—	—	—
10	4	1	—		—	—	—	—	—	—		—	—	—
270	120	40	30		30	21	20	25	25	9		20	21	40
—	—	—	—		—	—	—	—	—	—		—	—	—
—	—	—	—		—	—	—	—	—	—		—	—	—
4	3	1	—		2	—	—		—	—		—	—	—
—	3	3	3		45	30	60	78	84	36		9	—	1
—	—	—	—		—	—	—	—	—	—		—	—	—
2	—	—	—		8	30	3	20	2	1		—	—	—
—	—	—	—		—	—	—	—	—			—	1	—
9	2	4	4		1	—	—	—	—	—		—	—	1
7	6	3	4		6	6	6	1	3	—		1	—	3
—	—	—			1	—	—		—	—		1	1	3
—	—	—	—		—	—	—	—		—		—	—	—

the blood of 15 healthy carriers in June and July 1941.

July 1941

17	18	19	20	21	22	23	24	25	26	27	28	29	30	1
—	1	—	—	—		—	—	—	—	—	—		1	—
—	—		—	—		—	5	—	—	—	—		—	—
—	—	—	—	—		—	—	—	—	—	—		—	—
20	102	25	15	40		90	30	45	72	60	204		210	108
—	—	—	—	—		—	—	—	—	—	—		—	—
—	2	—	2	—		—	1	1	—	—	—		—	—
1	—	2	—	—		—	—	2	1		2		1	1
12	2	—	—	—		1	—	—	2	2	1		—	6
—	—	—	—	—		—	—	—		—	—		—	—
—	2	1	22	42		156	138	132	84	7	5		—	—
—	—	—	—			—	—	—	—	—	—		1	—
—	—	—	1			1	6	9	8	2	7		5	—
2	1	1	3	1		5	2	3	—	10	1		2	—
—	—	—	1	—		—	—	—	—	—	—		—	—
—	—	—	—	—		—	—	—	—	—	—		—	—

initially increased (parasite relapse) and subsequently dropped down to zero again (see fig. 1—3).

Among the 6 exceptions 5 children occurred, who as early as January had got completely the better of their parasites (test usually negative) and a spleenless boy, whose case will be discussed later on (test always positive).

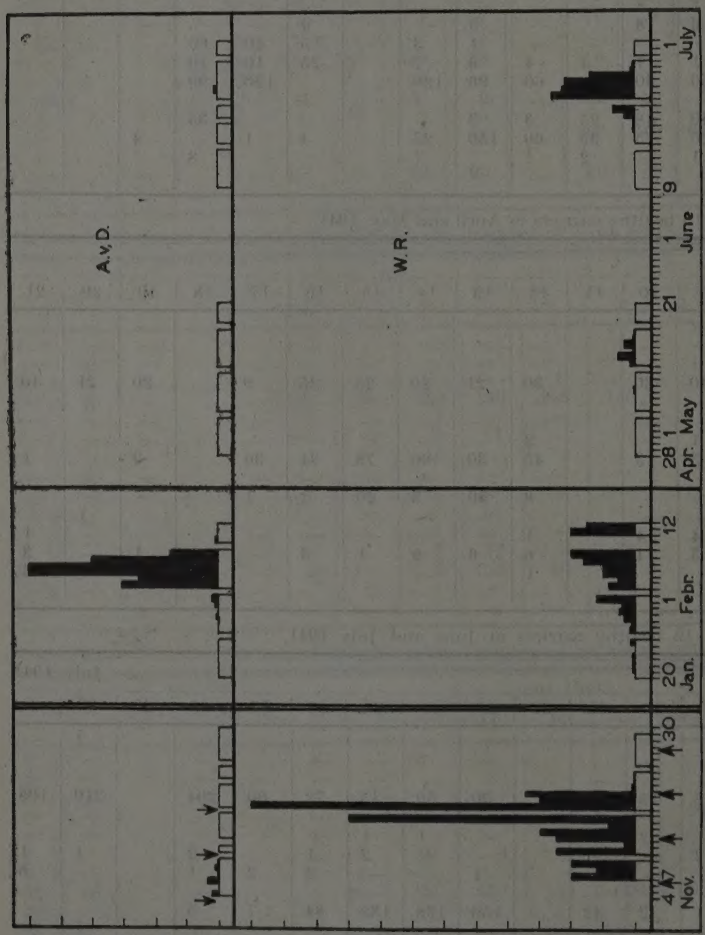

Fig. 1. Healthy carriers (A. v. D. and W. R.) during four periods of 3½ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

The length of the periods in which an uninterrupted series of positive results was obtained ('positive phases') varied in January/February between 11 and 16 days (11, 12, 13, 14 and 16 days; average 13 days), in April/May between 6 and 13 days (6, 8, 12 and 13 days; average 10 days) and in June/July between 9 and 11 days (average 10 days). So the 'positive periods' tended to grow

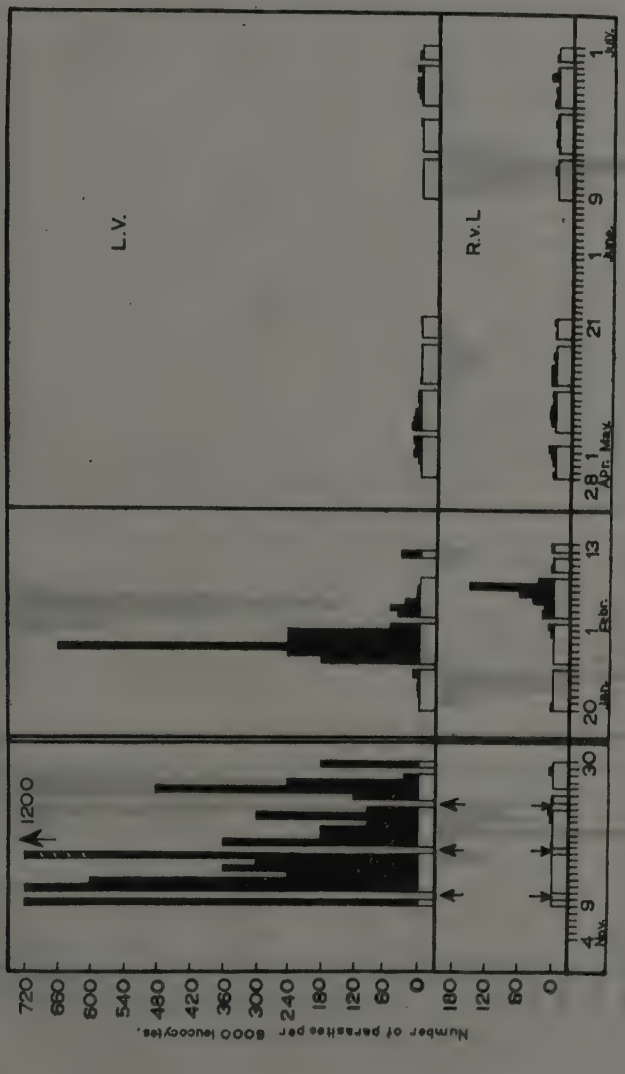

Fig. 2. Healthy carriers (L. V. and R. v. L.) during four periods of 3½ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

shorter during the period of observation. Moreover, the tops came down gradually to a lower level; in two carriers only (W. v. Z. and W.R.), the data of whom are represented in the graphs, we noted higher numbers in June than in May.

During the period of general decrease in number of the circulating parasites between January and July, the peripheral blood of several

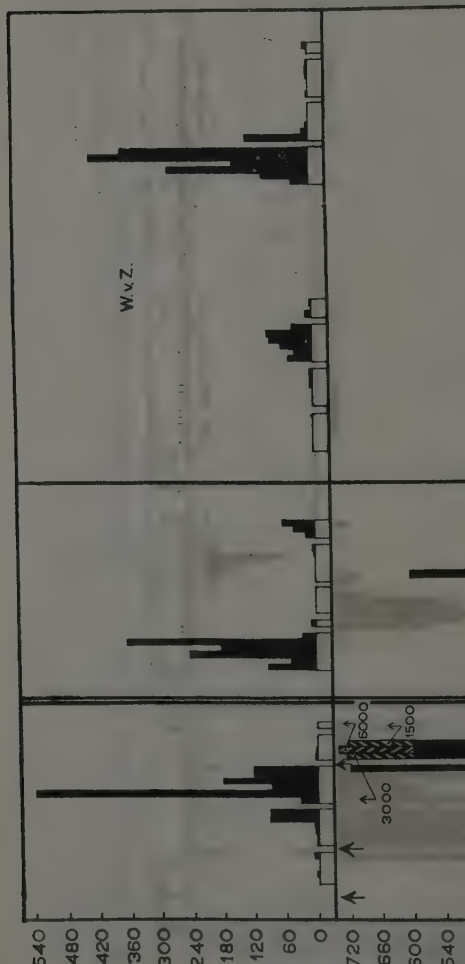

Fig. 3. Healthy carriers (W. v. Z. and D. de R.) during four periods of 3½ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

carriers gradually grows free from parasites. So out of 9 carriers, in whom in January still fairly significant numbers of parasites had been noted, showing, moreover, the alternating '+' and '—' phases (the spleenless boy thus left out of consideration) in May 7 and in June only 6 were left. In one of these (R. v. L.) as early as May the periodicity had changed into a practically continuous presence of very few parasites (1—10 per 6000 leucocytes).

The length of a 'negative phase' is known to us from the January/February series only. In three carriers we could note in the beginning of the observation period of 3½ weeks the ending of a '+ wave' and, at the close of the period, the beginning of a new one. The duration of the 'negative' intervals was 8, 10 and 14 days (average 11 days). In the later series never any 'negative phase' limited to a period of 3½ weeks could be noted. The chance of its occurrence may well have been diminished by the already mentioned decrease in number of the carriers. We can hardly surmise, however, that this would be the only reason for the non-occurrence of complete 'negative phases' in May and June, as in this period several positive periods did occur. So we are inclined to suppose that between January and July, along with the slight shortening of the '+' phases, the '—' phases have increased in length; as a matter of fact this will diminish the chance of noting its beginning and its end in one and the same observation period. Whether an inverted ratio exists between the length of the negative and positive phases, *i.e.*, whether the intervals between the tops of the '+' phases are of equal length, is an interesting question, which, however, cannot be answered without further investigation.

With regard to epidemiological investigation these facts show that (at least between January and July) the actual number of parasite carriers in a population-group can never be discovered by preparing a single blood film for each member, because part of the members of such a group will just be in a 'negative phase'. Table VI D shows the percentage of the actual carriers which would have been traced in our series by means of a single test.

Table VI

	Jan./Febr.	April/May	June/July
A. average number of parasites (per 6000 leucocytes) counted during the positive phases	62	22	46
B. maximum number of parasites per 6000 leucocytes	660	270	270
C. % positive results	60	36	30
D. % of the carriers which would have been traced in one day	61	40	36

Daily examination of all concerned would be far too laborious for an investigation in the epidemiological field. In accordance with the presented data on the periodicity of the parasite relapses, it may, however, be claimed as sufficient to carry out a second and a third blood test about 10 and 16 days after the initial one. By these means, only such carriers will escape their being traced by the investigator in whom, fairly rarely, slight numbers of parasites can be detected and who as such are of little significance for the final result.

As in the various carriers the tops do not occur simultaneously, changing metereological conditions cannot provide an explanation for the described periodicity. It might be considered, however, as an image of the struggle between parasite and host. After each revival of the activity of the parasites in the reticulo-endothelial tissue, (which may give rise to a 'parasite relapse' in the periphery) a certain reserve of specific counteracting force seems to have originated (negative phase). In the course of a limited number of days, however, this defensive reaction decreases in such a measure, that the parasites may come into play again. After some months the periodically revived activity of the parasites in their hiding-places has apparently weakened in such a degree that it can only manifest itself in the peripheral circulation, when it is at its height: then during a few days a small number of parasites will occur in the blood (short and low '+' wave).

A spleenless carrier of parasites

Among the six carriers in whom the periodical appearance and disappearance of the parasites was less distinct than in the others occurred a boy of eight years (W. de R.) whose spleen had been removed in March 1938 on account of a trauma. In May 1938 he had his first attack of malaria. Thus he had probably been infected in autumn 1937 and may well have been a carrier of parasites since May 1938, as he had since been complaining of many accesses of fever. If we leave the November series (during the quininisation) out of account for the moment, we note that in him the result of the blood test has never been negative (fig. 4). It is true that the curve for the numbers of parasites in his blood shows distinct rises and falls, but whilst for other carriers the 'waves' from January to July generally either decrease markedly in height or disappear completely and at the end of each decrease in number the zero line is reached again, in the spleenless boy the imaginary line which would connect the tops of the 'waves' descends but very slowly and every day parasites could be detected in his blood.

The fact that for him the tops in January were of about the same height as for the other carriers may not be considered as of much value. Indeed the spleenless boy had probably been carrier during a much longer period than most of his companions in infortune.

When in November 1941 in all 15 carriers one more blood test

was made, the spleenless boy was one of the two in whom parasites were traced and even in a fairly high number(100/6000 leucocytes). Whilst many had vanquished their germs completely — or nearly so — in July (*viz.*, in most of the cases about a year after their initial attack of malaria), the spleenless boy had not yet attained this stage in probably $3\frac{1}{2}$ years after the first access of malarial fever. We need not wonder at this, as it is well known, that the spleen is specially active in destructing malaria parasites. According to TALIAFERRO (5) it is, moreover, the field of action of a specific antibody, causing an opsonic activation of macrophagocytes.

The fact, that even in the absence of the spleen the tops of the curves do not surpass a definite height in the end, shows that even under these conditions the body may defend itself against the parasites and eventually free itself from them (*e.g.* by means of the remaining part of the reticulo-endothelialsystem).

It was, moreover, remarkable that during the autumnal quininisation the blood test in the spleenless boy remained negative for a long time without interruption. This does neither agree with results, obtained in him in later series, nor with those in most of the other carriers of parasites in the period in which they took quinine. Possibly parasites occurring in the spleen are less vulnerable for quinine than those which are hiding in other organs, *e.g.* in the bone-marrow or in the liver. Another possibility lays in the assumption that the circulating parasites, which are damaged prima-

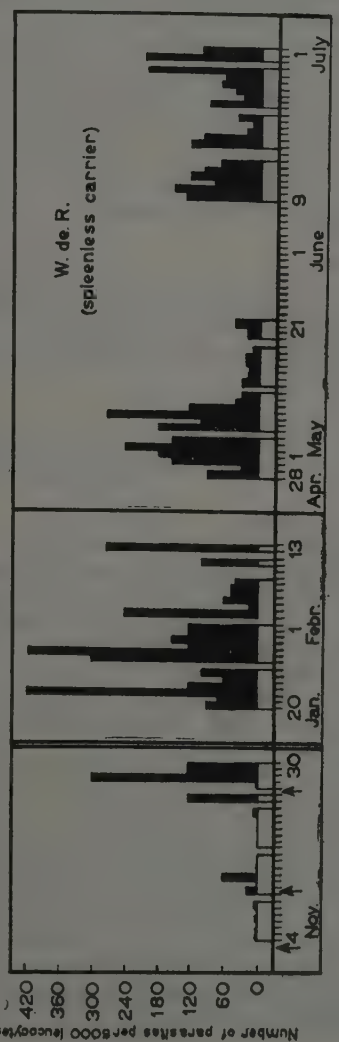

Fig. 4. A spleenless carrier (W. de R.) during four periods of $3\frac{1}{2}$ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

rily by the quinine, are more swiftly replaced in a person with a larger store of parasites.

III Stages of development of the parasites.

All forms, also gametocytes (especially male) occurred up till the end of the observation period. No change in the ratio of their occurrence could be noted.

The tertian periodicity remained recognisable and was most distinct in the April/May series, in which as a rule we noted during the '+' phase every other day an alternation of mostly young with mostly full grown rings. As, moreover, the schizonts broke into a normal number of merozoites, it may be supposed that the reproductional process is not influenced disadvantageously by the defensive action of the carrier. Other investigators are of the same opinion (cf. Taliaferro (5)).

IV The state of health of the parasite carriers.

Our observations remained restricted (i.a. by the insufficient cooperation of those concerned) to the subjective health statements, the body temperature and the report of the school physician.

The connection between the seriousness of the complaints and the number of parasites in the blood

1. In carriers of parasites who felt in perfectly good health and complained at most sometimes of a headache, we counted 960, 2400 and 2520 parasites per 6000 leucocytes.
2. 'Seedyness' and not visiting school (without calling in medical advice) we noted to occur along with 180, 3600, 4500 and 6060 parasites per 6000 leucocytes.
3. Carriers who felt ill and who, notwithstanding this, did not seek medical advice and took quinine on their own authority had 3900, 4920, 6000, 9000 and 9030 parasites per 6000 leucocytes in their blood. Sufferers from malaria with as high a number of parasites thus may remain untraced, since as a rule they do not seek medical advice even in a relapse, as long as they are still provided with quinine. Moreover, such a treatment with quinine on their own initiative mostly lasted for a few days only! As most of the numbers mentioned here bear on autumn and as this is also the season in which the mosquitoes transmit the malaria parasites, it is not to be wondered at that the disease in the population group observed by us has maintained itself over a long period.
4. When they felt ill in such a degree that medical advice was sought for, the numbers of parasites varied between 6000 and 24000 per 6000 leucocytes.

The body temperature of the carriers

In the summer of 1940 the blood of several carriers was tested daily during 7 to 10 days and as far as possible their temperature, taken rectally, registered. When more than 240 parasites per 6000 leucocytes were counted, a rise of temperature was always noted; lower numbers often occurred along with a perfectly normal temperature. On the other hand we could note twice a rise in temperature up till 38° C. during a short period, whilst not a single parasite could be traced in the peripheral circulation; 2 or 3 days after the rise in temperature, however, parasites occurred again in the blood in both cases and subsequently increased greatly in number.

The report of the school physician

From none of the children concerned special complaints had come in during the period of our observation.

In fact the sufferer from chronic malaria escapes in a number of ways the attention of his surroundings and of those whose task it is to combat the disease. Not only any typical symptoms are usually absent, but, moreover, the result of the blood test is often over a long period negative and many complaints, even those of a fairly serious character, do not reach the physician. As a consequence, the term 'healthy carrier' may not be entirely justified, but it is clear now, why it has become a generally accepted one in the course of time.

Summary

In 1940 and 1941 observations were made on the number and mode of appearance of parasites of benign tertian in the blood of 'healthy' carriers of parasites. The results of this investigation indicate that during the period of incubation shortly before the attack no or at most a few parasites occur in the peripheral circulation; this agrees with the experience of others.

The group of carriers who had already suffered from an attack was studied; in this country these are the main source of infection for the mosquitoes. It appeared — at least between January and April following the year in which they had fallen ill — that as a rule periods in which the blood test is negative alternate regularly with others in which the number of parasites first increases (parasite relapse) and subsequently drops down to zero again. The length of the negative and that of the positive phase averages about 12 days.

No parasites — or only a few of them — are to be found in the blood of most carriers one year after the outbreak of the disease. This was not the case in a spleenless carrier even after 3½ year; moreover, the blood test of the latter was never negative.

The tertian periodicity remained recognisable up till May 1941. Some data were collected on the general state of health and the body temperature of the carriers. It may happen that a person with 2400 parasites/6000 leucocytes still has the impression of being in quite good health.

An attempt to induce the disappearance of the parasites from the peripheral circulation by means of autumnal quininisation in a population group is described; the results, however, were disappointing.

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VIBRIO RESEARCH IN THE HEJAZ IN CONNECTION WITH THE EL TOR PROBLEM

by

R. ABDOELRACHMAN

(Received March 25, 1944).

1. THE HISTORY OF THE EL TOR PROBLEM.

It was ROBERT KOCH, who rendered a great service to mankind in discovering *V. cholerae* as the originator of the cholera asiatica in 1883, which fact offered the possibility to take appropriate hygienic and prophylactic measures against this very infectious disease. He succeeded in cultivating vibrios out of the stools of patients suffering from cholera and out of those of people, in whom the disease proved fatal. These micro-organisms proved morphologically, culturally as well as serologically specific. He also stated, that people by drinking water containing a large amount of these vibrios might be infected by the disease.

Later on investigators of many countries could isolate out of the water of numerous rivers, wells and sources vibrios, which differed in some respects from the cholera vibrio of KOCH and which therefore were reckoned to the group of water vibrios.

Moreover during many epidemics of cholera out of different surface waters vibrios were cultivated by means of the enrichment method of SCHOTTELIUS with peptone water. By means of the methods of research then available these vibrios could not be distinguished from *V. cholerae*, and so were qualified as such (24).

In those days only pathogenic *V. cholerae* and apathogenic water vibrios were known, organisms which by means of the bacteriological methods (biochemical test, agglutinating reaction of GRUBER and DURHAM, PFEIFFER's test) could be easily differentiated.

In later years cholera-like vibrios were isolated out of human feces, which were morphologically and culturally similar to *V. cholerae* but which were not agglutinated by highly agglutinating cholera serum (3).

In 1897 on a pilgrimship RUFFER cultivated vibrios out of the stools of a pilgrim returning from Mecca and who suddenly died; these bacteria were found to be quite similar to *V. cholerae*, although in the Hejaz, neither on the ship nor at the quarantine station El Tor cholera occurred.

So during some years for all returning pilgrims from Mecca, who

had died at this quarantine station, it was investigated whether vibrios occurred and autopsies were carried out on all corpses. This resulted in the isolation during five successive pilgrim seasons of many vibrios, which by means of the agglutinating test could not be distinguished from *V. cholerae* (26).

In 1905 GOTSCHLICH detected vibrios in 38 out of 107 people returning from Mecca, 6 of which were agglutinated by highly agglutinating cholera serum and 32 not. These vibrios were considered by GOTSCHLICH as real cholera vibrios, although there was no cholera in the Hejaz, neither on the pilgrimship nor at the quarantine station El Tor. The people in whom the vibrios were found were qualified by him as carriers of cholera vibrios (9).

RUFFER, who was president of the „Conseil sanitaire maritime et quarantenaire d'Egypte" did not accept this diagnosis and declared the 6 agglutinable vibrios as not identic with *V. cholerae*. He was of opinion, that the agglutination test was useful but not specific and a conclusion as to the specific nature of these El Tor vibrios might merely be arrived at after the problem had been viewed epidemiologically as well as bacteriologically. Because of the fact that the 6 pilgrims in whom the El Tor vibrios had been found had left their country already 2 or 3 months earlier and as cholera vibrios after about 2 weeks will have disappeared from the intestines of a patient (CRENDIROPOULO 1902), RUFFER thought it probable, that the pilgrims might have been infected with the vibrios at Mecca. Although RUFFER had only found inagglutinable vibrios and no agglutinable ones in the Zam-Zam water from Mecca, he supposed that this water, which is drunk by all pilgrims, was the source of origin of *Vibrio El Tor* (26).

So the El Tor problem had arisen, *viz.*, the problem whether *V. El Tor* is identic with *V. cholerae* (monistic point of view) or not (dualistic conception).

This question is not solved as yet; some investigators are of opinion that both vibrios are identic, as they have the same serological properties, others regard them as two kinds of vibrios, as they differ in action on blood plates and in blood broth and as moreover in recent years differences in the chemical structure of the two bacteria have been stated.

KOLLE and MEINICKE were the first investigators, who in 1906 supported the opinion of GOTSCHLICH (12).

In the same year KRAUS and PRIBRAM opposed the monistic conception for epidemiological reasons and because of the fact, that *V. El Tor* produced haemotoxine in blood containing media whilst *V. cholerae* lacked this property (13).

Later on MÜHLENS and VON RAVEN (1906) (23), NEUFELD and HAENDEL (1907), BAERTHLEIN (1914) (1), CLAUBERG (1921), MACKIE (1929) agreed with the monistic point of view; on the other hand LIEFMAN and NIETER (1906), KRAUS and TRANTSCHOFF (1906), KRAUS and RUSS (1908), KRAUS and FUKUHARA (1909), PFEIFFER (1908), VAN LOGHEM (1910) (18), GRAIG (1914), LÖWY (1915) (15),

KÄMMERER (1920), LAMPE (1922) (13) defended the dualistic conception.

In collaboration with STEENSMA (17) and later on with SNAPPER in 1918 (27) VAN LOGHEM ascertained the haemolytic action of *V. El Tor* in contrast with the haemodigestic property of *V. cholerae*.

As according to several investigators the haemolytic property was not only found for *V. El Tor* but also for *V. cholerae*, in 1922 LAMPE (14) ascertained that the *V. El Tor* haemolysine was formed very soon, that it was partly thermolabile and partly thermostabile and that it had an antigenic action (exohaemolysine), whilst the cholera haemolysine was formed later and had no antigenic character (endohaemolysine).

In 1931 once more some *V. El Tor* strains out of the feces of pilgrims not suffering from cholera were isolated by DOORENBOS. He is the only investigator who considered the *V. El Tor* as a *V. cholerae* infected with bacteriophage, so he is a supporter of the monistic theory (2).

VAN LOGHEM in 1933 pointed out emphatically the diagnostic value of properties of haemolysis of *V. El Tor* and of haemodigestion of *V. cholerae* (19).

In the same year FLU examined some cholera and El Tor strains biochemically and serologically. In these experiments 5 cholera strains, which 16 years earlier had shown no haemolytic action, now appeared to possess an although temporary haemolytic property. Because of this reason FLU, who was formerly supporter of the dualistic point of view, began to doubt it (5).

Meanwhile new serological methods of investigation were adopted and several investigators as BALTEANU (1926), SHOUSHA (1931), ZIMMERMANN (1932) (29), ABDOOSH (1932), GOHAR (1932), GARDNER and VENKATRAMAN (1935) (6), considered only the cholera O-serum as specific; but even using this specific cholera O-serum none of them except GOHAR was able to distinguish *V. El Tor* from *V. cholerae*.

According to GISPEN in 1938 the agglutinating property of *V. El Tor* would be more heat-stable than that of *V. cholerae* (8).

Other investigators tried to divide the vibrios according to their biochemical reactions or to their chemical structure.

HEIBERG examined in 1935 more than 400 vibrio strains by applying mannose, saccharose and arabinose media; so he arrived at 6 groups of vibrios, but *V. El Tor* and *V. cholerae* appeared to belong to the same group (10).

In 1932 LINTON and his collaborators analysed the vibrios chemically and found that they contained several combinations of 3 polysaccharides and 3 proteins; in this way he was able to classify the vibrios into 6 groups and so to distinguish *V. El Tor* from *V. cholerae* (16).

In the meantime in 1937—1938 an explosion of gastroenteritis

broke out in South Celebes (Macassar and its surroundings), which according to the examination of DE MOOR was caused by vibrios, which were in every respect quite similar to *V. El Tor*. The same vibrios were cultivated out of waters, which were probably polluted with the stools of the patients (21). The correctness of this diagnosis was confirmed at VAN LOGHEM's laboratory at Amsterdam. This was the first time, that *V. El Tor* was stated as the originator of a disease which resembled the cholera; this fact gave a new turn to the El Tor problem (20).

2. RESEARCH IN THE HEJAZ.

DE VOGEL, delegate of the government of Netherlands East India and member of the „Comité de l'Office international d'hygiène publique", suggested in 1935 that the Dutch government might have the possibility investigated of the transmitting of El Tor vibrios to and from Mecca. For this purpose control ought to be carried out in the countries of origin of the pilgrims and in the Hejaz itself.

In Netherlands East India this investigation was carried out by MEYER (22), whilst in the Hejaz the task was given to me.

This control was carried on from October 1937 till June 1938 along with my own extensive clinical work and in very difficult circumstances. During 9 months I examined 1109 stools of different people and 90 water samples out of various water sources. In view of the El Tor gastroenteritis in South Celebes special attention was paid to the pilgrims coming from this part of India.

The stools of healthy and sick people coming at the policlinic were controlled, namely 602 pilgrims from Netherlands East India, 310 Indonesians living at Mecca, 21 pilgrims from the Malay States, 113 Malays living at Mecca and 63 Arabs and people of other nationalities.

The control of the feces and the waters was done by means of the enrichment method of SCHOTTELIUS with the Dieudonné blood plate and further by applying the common bacteriological tests.

Vibrios were isolated out of the stools of 11 persons, 2 of whom were pilgrims freshly arrived (1 from South Celebes), 7 were Indonesians from Mecca and 2 were negroes living as well since a long time in this country. These vibrios were motile, Gram negative, they fermented glucose, maltose, mannose and saccharose, did not ferment lactose, formed a bright pink surrounding area on the goats blood plate, haemolysis in goat blood broth, gave a positive cholera-red test and were agglutinated by cholera-OH-serum till a titre varying from 1 : 400—1 : 3200.

According to a further investigation at Amsterdam and at Batavia they were not agglutinated by cholera-O-serum and therefore were neither *V. cholerae* nor *V. El Tor*.

In connection with the water control as to the occurrence of vibrios some information about the water supplies in the Hejaz may be presented.

The people in this part of Arabia derive their drinking- and

bathing-water only from the few wells and sources in the oasis and in the lower parts of the country and by gathering the rarely falling rainwater. Most of those wells exist since hundreds of years and some of them are regarded by the inhabitants as holy. The most important and holy well is the Zam-Zam well in the central mosque at Mecca, the water of which is drunk by all pilgrims coming there. The Ain Zoebaida for Mecca is the longest and the best waterchannel, it has been built about 700 years ago on order of queen Zoebaida. In more recent years at the harbourtown of Djedda condensed sea water is also to be had, which is the best drinking water in this town.

I examined most of the water sources in the Hejaz as to the occurrence of vibrios namely:

29 samples	Zam-Zam water	} Mecca
9 samples	Zoebaida-channel water	
3 samples	Wasirya-channel water, Djedda	
4 samples	Sarga channel water, Medina	
45 samples	out of the other water wells and sources	
90 samples	totally	

VAN ROMBURGH in 1886 had already examined chemically the Zam-Zam water which he had obtained from SNOUCK HURGRONJE. This water was clear, contained no ammonia and a negligible quantity of organic substance (25). The chemical composition of 1 liter of this water was the following:

Cl	0.5563 gram
SO_3	0.3955 ,,
N_2O_5	0.7255 ,,
CO_2	0.3170 ,,
SiO_2	0.4720 ,,
CO	0.4120 ,,
MgO	0.1520 ,,
Na_2O	0.5776 ,,
K_2O	0.2719 ,,

A second examination of 0.25 l of the water gave the following results:

$CaSO_4$	0.2501 gram
$MgSO_4$	0.1140 ,,
Na_2SO_4	0.3307 ,,
K_2SO_4	0.1257 ,,
SiO_2	0.0118 ,,
Totally	0.8323 gram residu

Moreover the water had a particular metallic taste, which is known to me by own experience.

According to some European travellers, such as BURTON and BURCKHARDT in 1829 and also according to the inhabitants of Mecca the Zam-Zam water has a laxative action, when drunk in a large quantity.

The first investigator who has examined the water bacteriologically was RUFFER in 1907 (see above).

In 1928 VAN DER HOOG stated, that the Zam-Zam water did not contain ammonia and nitrates but only nitrites; the coli fermenting test was positive in 5 cc, the number of bacteria was 1880 in 1 cc (11).

From 12 out of the 29 samples of Zam-Zam water I cultivated vibrio strains, one of which appeared to be identic with *V. El Tor*, according to the examination at Amsterdam as well as at Batavia.

The 11 other strains were agglutinated by cholera-OH-serum but not by cholera-O-serum; therefore they were neither *V. cholerae* nor *V. El Tor* and merely water vibrios.

Out of the 61 water samples derived from the other wells, sources and channels only 4 vibrio strains were isolated, which also appeared to be water vibrios.

So the results of my examinations of stools and water samples in the Hejaz were, that several times water vibrios were found, especially in the Zam-Zam water and that once a vibrio strain was cultivated, which was in every respect identic with *V. El Tor*.

This investigation was carried out in a period when no cholera occurred in the Hejaz. Thousands of pilgrims had drunk the Zam-Zam water without any harmful effect.

3. RESEARCHES ON VIBRIO CARRIED OUT BY OTHER INVESTIGATORS.

The results of MEYER's investigation in 1936 at Batavia for 20444 persons and those of SARDJITO at Semarang in the same year for 2875 people and 40 water samples as to the occurrence of *V. El Tor* were negative (22).

Neither in Egypt was *V. El Tor* found, either in stools or in waters (3).

After GOTSCHLICH in 1905 and DOORENBOS in 1930, the latter isolated again in 1931, 1932, 1933 and 1934 at El Tor, El Tor vibrios out of the stools of home-bound pilgrims (4).

In 1933 SHAHIN PASCHA examined 1532 Egyptian pilgrims on their outward as well as on their homeward voyage. Whilst no *V. El Tor* was found before they went, it appeared that home-bound 8 of them carried these vibrios.

SUHBI WAHBI in 1938 examined home-bound Irak pilgrims at the landquarantine station Najaf and found in the feces of 1 out of 61 persons El Tor vibrios (28) and according to GILMOUR in 1938 there were found 8 El Tor vibrio carriers among the home-bound Egyptian pilgrims (7).

Summary and conclusions.

In 1883 ROBERT KOCH discovered *V. cholerae* as originator of the cholera asiatica; this important discovery has enabled us to conquer the very infectious and dangerous disease. Subsequently water vibrios were found in many water sources; these microbes

can easily be differentiated from *V. cholerae*. Several times *V. cholerae*-like vibrios were isolated out of the stools of people suffering from gastroenteritis.

In 1905 GOTSCHLICH and afterwards other investigators as well isolated at El Tor out of the feces of pilgrims who were not suffering from cholera, vibrios which according to some of them were identic with the actual *V. cholerae*, but which in the opinion of others such as RUFFER must not be considered as such.

DE MOOR cultivated in 1937—1938 at Macassar and its surroundings out of the stools of people suffering from a cholera-like gastroenteritis as well as out of waters, which were probably polluted with the feces of those people, germs which in every respect were like the El Tor vibrio.

Finally I detected in 1937—1938 in a water sample derived from the Zam-Zam well at Mecca (Hejaz), the water of which is drunk by thousands of pilgrims without any harmful effect, germs which were absolutely identic with *V. El Tor* of GOTSCHLICH.

The El Tor vibrio is never found in the stools of people bound for the Hejaz, but several times in the feces of home-bound pilgrims.

These facts make me tend to the following conclusion:

I. *V. El Tor* forms a group of vibrios of its own, neither identic with *V. cholerae* nor with the water vibrios.

II. This group of vibrios may be subdivided into 2 types:
1°. The apathogenic type, which is found in the Hejaz and surroundings; this vibrio may be called: *Vibrio El Tor*, type Hejaz.
2°. The pathogenic type, which is found at Macassar and surroundings: this may be called: *Vibrio El Tor*, type Macassar.

Literature.

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THE ACTION OF MANGANESE ON THE DEVELOPMENT OF *ASPERGILLUS NIGER*

by

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1. Introduction.

The reason why an attempt has been made to compose a manganese standard with *Aspergillus niger* as indicator is the following.

A plant disease exists, known in this country as „Veenkoloniale haverziekte" (grey-speck disease, Dörrfleckenkrankheit), which is apt to occur on sandy humous soils as the result of too strongly alkaline manuring. As oats are particularly sensitive for this disease, this crop plant has been the first in which its symptoms have been noted (Sjollema and Hudig (20)). Most markedly under influence of a sudden raise in temperature the leaves will present greyish dry spots and the tip parts of the leaves will fall over with a sharp kink at the necrotic region. The further development will stagnate and the ultimate yield will be influenced very unfavourably. An acid manuring will prevent the further occurrence of this disease, but a more direct although not lasting result may be attained by a dressing with mangano sulphate or other manganese compound.

So the question arose how to explain the favourable action of manganese.

The fact that manganese might range among the elements essential for the normal development of plants has been suspected already a long time ago. In 1909–12 Bertrand (1, 2, 3) has pointed to the beneficent effect on plant development of small gifts of manganese to the soil. Mazé (13) working with corn plants in highly purified solutions notes a weaker development and a chlorose in the leaves in the absence of manganese. McHargue (14) could induce definite deficiency symptoms in the foliage of peas, when grown in a solution deprived of manganese. An important investigation of also distinct agricultural value was carried out by Samuel and Piper (19) in South Australia. These scientists succeeded in inducing in oats, grown in culture solution made up with very pure chemicals and free from any manganese, symptoms of a similar nature as those of the grey-speck disease. When, however, 0.25 mg per liter had been added to the solution, healthy

plants developed. When the content of Mn in healthy and diseased plants grown in the field was compared, it was in the latter markedly lower than in the former; whenever the content fell below a definite level the symptoms of the disease were apt to occur. So the connection between the presence of plant-available manganese and a healthy plant development has been well-established. More recent research may have shown that the connection is of a more complicated nature than originally surmised (GERRETSEN (8)), the fact that manganese is an essential element is left unshaken.

It has been tried along several lines to estimate the content of plant-available manganese in the soil. For a recent survey of the literature on this subject the reader is referred to WIERINGA (28).

As has been stated already, grey-speck disease occurs mainly in alkaline soils not below pH 6.7. Manganic compounds vary greatly in solubility, depending on the presence either of mangan*i* or mangan*o* ions. For the ascertainment of plant-available Mn the determination of the total amount in alkaline soils is of no value. PIPER (18) arrives at the conclusion, that its solubility will depend: 1° on the pH of the soil: under alkaline condition mangan*o* ions being oxidised to mangan*i* and thus insoluble; 2° on the redox potential: under reducing conditions mangan*i* will be reduced to mangan*o* and so the solubility will increase. Later LEEPER (10) could ascertain the existence of healthy alkaline soils, which even at a high redox potential will furnish a leachate high in Mn when quinol has been added to the leaching solution. For sick alkaline soils, however, the addition of quinol will not involve an increase of Mn in the leachate. Apparently the mangan*i* may exist in various levels of activity; when most active it will oxidise other compounds easily and will be reduced to soluble mangan*o*. As the amount of soluble manganese is in such a large measure dependent on experimental conditions and it is thus difficult to estimate the actual amount available to the plant, it seemed worthwhile to investigate whether *Aspergillus niger* might be made to serve this end.

In fact experimental results of many scientists indicate that *Aspergillus niger* assimilates several elements along similar lines as higher plants. An *Aspergillus* standard is in use for the estimation of the available amount of K (NIKLAS (17)) and P (NIKLAS (17), GERRETSEN and BLUMENDAL (9)); in these cases the yield in weight of the fungus for graded amounts of these substances has been determined and thus a standard composed. By MULDER (15, 16) *Aspergillus niger* has been made to serve the estimation of Cu, the shade of colour of the spores marking here the distinction between the various grades of the scale. For Mg (MULDER (15, 16), SMIT and MULDER (21)) it is the density of growth of sporangia which marks the various grades.

The essentiality of Mn for the full development of *Aspergillus niger* has been ascertained by several investigators. BERTRAND

(4, 5) as early as in 1912 succeeded in composing a culture solution by means of which he could provoke a difference in yield of mycelium between the solution completely lacking in Mn and a solution which had been provided with 0.1 γ Mn per liter. To attain this result the chemicals had been very rigidly purified by a threefold recrystallization and the fungus had been grown in quartz vessels. In the absence of manganese no sporangia had developed.

STEINBERG (23) presents in 1935 experimental results attained at in the absence of manganese. He frees his culture solution from traces of heavy metals by means of adsorption on $CaCO_3$. He states: „omission of manganese results in the development of intensely white felts consisting of individual and but partly coalesced colonies. The brittle felt is as a result perforated by numerous holes. Spore formation, moreover, is sharply reduced or even suppressed in the absence of manganese. Suppression of spore formation in cultures containing a limited amount of Mn has been noted at yields 3/4 maximum".

So an attempt to compose a graded scale for the estimation of manganese by means of *Aspergillus niger* might prove to be well-founded.

2. EXPERIMENTS.

The experimental technique for the composition of a graded scale is very simple. It consists in the cultivation of *Aspergillus niger* in a complete culture solution, the only varying factor consisting in the amount added of the element studied. When for definite amounts of such an element definite responses in the development of the mould occur, such a range in amounts may serve in its turn as a standard for the estimation of unknown amounts of the element in question.

A culture solution such as it has been used by MULDER was employed. Its composition is:

A	B
50 g glucose	20 mg $FeCl_3 . 6 H_2O$
5 g KNO_3	20 mg $ZnSO_4 . 7 H_2O$
2.5 g K_2HPO_4	1 mg $CuSO_4 . 5 H_2O$
1 g $MgSO_4 . 7 H_2O$	1,5 mg $Na_2MoO_4 . 2 H_2O$
1 l dist. water	3 mg $MnSO_4 . 4 H_2O$

The finding of a suitable source of iron offered difficulties. The $FeCl_3$ which had been used by MULDER proved not free from Mn; various other iron salts qualified as of high purity were tested, but no clear-cut deficiency symptoms in the absence of Mn could be provoked. Prof. TENDELOO had the kindness to prepare out of chemicals of the highest grade of purity $FeSO_4 . 7 H_2O$, which served my aim perfectly. I am much endebted to him for this help. So all cultures were provided with 85 mg $FeSO_4 . 7 H_2O$ per liter, equivalent to 20 mg $FeCl_3 . 6 H_2O$.

Initially the culture medium was prepared along the same lines as MULDER did, *viz.*, after the chemicals A had been added 5 g coal (norite) per liter was added, the suspension shaken for 5 minutes, filtered through a Buchner filter and supplied with the chemicals B, the oligopleronts. This purification by means of norite, however, did not serve, like this had proved the case in a study on molybdenum (16). The filtrate proved not free from Mn, the norite apparently setting free some trace of manganese. So the method followed by STEINBERG (24) was adopted. 15 g $CaCO_3$ per liter was added to solution A, which was sterilized during 20 minutes at 120° C. The $CaCO_3$ was filtered of on a Buchner filter and the oligopleronts were added.

1 liter Erlenmeyer flasks of Jena glass, which had been frequently in use, were used as culture vessels and were supplied with 40 ml of the medium. All vessels, when not stated otherwise, were put up in duplo. The chemicals used were of the highest grade of purity available. Before use all glassware was rinsed with 10% HNO_3, twice with tapwater and finally with distilled water. The distilled water was prepared in an apparatus out of Jena glass. The strain of *Aspergillus niger* used by MULDER was used throughout. Fresh cultures always served for inoculation. The cultures were inoculated with 5 drops taken from a suspension of spores in distilled water. After incubation at 28° C. during 5 days the development of the fungus was ascertained. When the weight of the yield was to be estimated the mat of fungus was removed out of the flask, rinsed in water and dried at 105° C. The duplos were generally weighed together.

STEINBERG (25) in an investigation on molybdenum has established that this element is essential for the full development of *Aspergillus niger* provided nitrate has been supplied as only source of nitrogen; when, however, an ammonium salt acts as such, no deficiency symptoms occur in the absence of molybdenum. He draws the conclusion that molybdenum may be an essential factor in the reduction process of nitrate to ammonium, in which form the nitrogen will be ultimately assimilated by the fungus.

In the physiology of higher plants BURSTRÖM (7) and LUNDEGAARDH (11) present data for manganese proving that the assimilation of nitrate by roots of oats is suppressed in its absence.

So in view of the possibility that similar phenomena might occur in the case of *Aspergillus niger* I started my experiments by comparing the effect of the absence of Mn in solutions provided either with KNO_3 or NH_4Cl in amounts equivalent for N. The results, however, appeared to be quite the opposite of those arrived at in the absence of Mo. Whilst the solution with NH_4Cl without any intentionally added Mn gave rise to white felts quite free from sporangia, the KNO_3 solution presented a dense growth of sporangia; visually such a culture could hardly be distinguished from one on a medium provided with Mn.

When the final pH of the culture with NH_4Cl was estimated

by means of Lyphan paper, it appeared to have sunk to 1.6. In the nitrate series the final pH was about 4.5. So the suppression of the growth of sporangia might either be due to the absence of Mn or to the low final pH of the culture solution. When, however, Mn had been added to the solution with NH_4Cl, sporangia developed, notwithstanding the pH sank at a same rate: the acidity as such apparently did not prevent their development (Fig. 1). The optimal development differed, however, somewhat from the development under less acid conditions, the sporangia occurring in tufts evenly scattered with uncovered regions between. Moreover when an ammonium salt solution had been buffered by a supply of $CaCO_3$ [1]), sporangia developed quite abundantly in the absence of Mn.

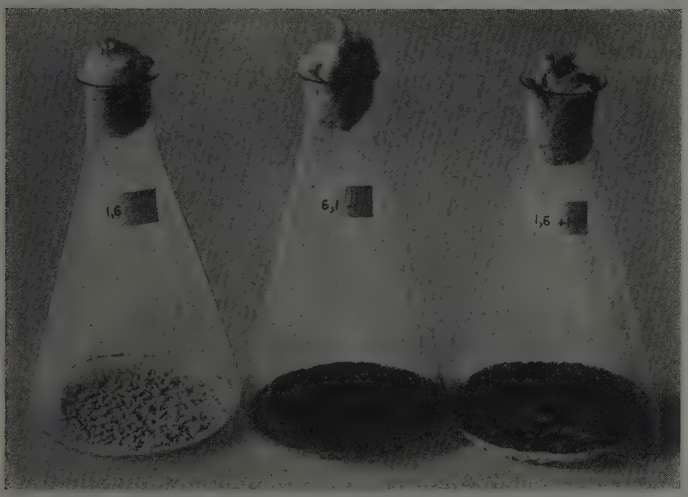

Fig. 1. $(NH_4)_2SO_4$ [2]) as source of nitrogen.
flask 1: — Mn — $CaCO_3$
flask 2: — Mn + $CaCO_3$ (0.5%)
flask 3: 25γ Mn — $CaCO_3$

The suppression of sporangia as a symptom of manganese deficiency could apparently merely be induced under strongly acid conditions.

So obviously the effect of acidification of a nitrate solution had to be tested as well. Phosphoric acid prepared from chemically pure anhydrous P_2O_5 was supplied for this purpose. The concen-

[1]) This $CaCO_3$ had been prepared out of chemically pure $CaCl_2$ and a solution of Na_2CO_3 which had been purified by adsorption on $CaCO_3$ in the same way as this was done for the nutrient solution.

[2]) $(NH_4)_2SO_4$ and NH_4Cl act similarly.

tration of H_3PO_4 actually used was 3.21 n, when titrated with thymolphtaleine as indicator. Whenever in the following volumes of H_3PO_4 will be mentioned, this will bear on 3.21 n H_3PO_4.

The size of the volume of H_3PO_4 to be added had to be tried out experimentally. On the one hand a too strong acidification will not allow of an initial development and on the other hand in a weakly acid solution the alkaline substances resulting from the metabolic processes of the fungus will prevent the occurrence of a final very low pH. A suitable volume having been asce tained and the final pH not surpassing about 2.5, no spo.angia developed (Fig. 2). Again when such an acid solution had been provided with some Mn, sporangia developed. The optimal development of the sporangia occurred more or less in tufts like in the medium with the ammonium salt. Here as well acid conditions appeared to be indispensable for the suppression of sporangia in the absence of manganese.

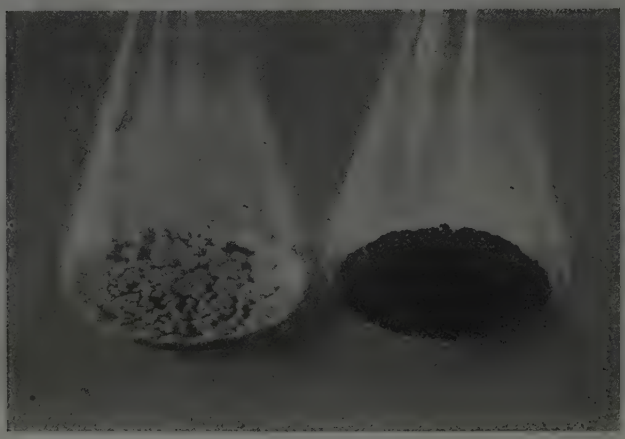

Fig. 2. KNO_3 as source of nitrogen.
flask 1: — Mn + H_3PO_4 (2.5 ml 3.21 n)
flask 2: — Mn — H_3PO_4

Once the method established for preparing cultures free from Mn which would show clear-cut deficiency symptoms, the way was open for the investigation into the possibility of composing a graded scale for manganese.

a). Nitrate as a source of nitrogen.

The initial experiment was carried out with KNO_3 as source of nitrogen, 2.5 ml H_3PO_4 being added to the individual cultures. In Table I Exp. 1 and 2 the results are presented.

Table I. Medium with KNO_3. Exp. 1 and 2 with glucose, Exp. 3, 4*a* and 5*a* with sucrose, Exp. 4*b* and 5*b* with sucrose and recrystallized KNO_3.

Exp.	Per culture		pH		Sporangia	Yield in g	Yield as % of (— Mn) cultures
	ml H_3PO_4	γ Mn	initial	final			
Acidified medium							
1	2.5	0	1.6	1.6	—	0.765	100
	2.5	0.1	1.6	2.3	+	0.720	94
	2.5	0.2	1.6	2.5	+++	0.640	84
	2.5	0.4	1.6	2.4	++±±	0.655	86
	2.5	0.8	1.6	2.5	++++	0.670	88
2	2.5	0	1.6	2.2	—	0.863	100
	2.5	0.05	1.6	2.2	±	0.857	99
	2.5	0.1	1.6	2.2	+	0.675	78
	2.5	0.2	1.6	2.3	+++	0.676	78
	2.5	1.0	1.6	2.3	++++	0.642	74
3	2.5	0	1.6	2.5	—	0.85	100
	2.5	0.005	1.6	2.5	—	0.83	98
	2.5	0.01	1.6	2.5	—	0.82	97
4*a*	2.5	0	1.6	2.2	—	0.83	100
	2.5	0.005	1.6	2.2	—	0.75	90
	2.5	0.01	1.6	2.2	—	0.72	87
4*b*	2.5	0	1.6	2.2	—	0.82	100
	2.5	0.005	1.6	2.2	—	0.77	94
	2.5	0.01	1.6	2.2	—	0.85	104
Non-acidified medium							
5*a*	0	0	6.4	5.2	+++	0.79	100
	0	0.005	6.4	5.2	++±±	0.885	112
	0	1	6.4	5.2	++++	0.915	116
5*b*	0	0	6.4	5.2	++±±	0.755	100
	0	0.005	6.4	5.2	++±±	0.795	105
	0	1	6.4	5.2	++++	0.787	104

— = complete suppression of sporangia; ++++ = optimal development.

In the absence of any intentionally added manganese sporangia are completely lacking; a minute dose of 0.05 γ induces a slight growth of sporangia, 0.1 γ allows of a slightly denser growth, whilst a culture with 0.4 γ is hardly to be distinguished from optimal development. These differences, however, covering merely such a small range, could not serve the purpose of a manganese standard.

In Exp. 3–5 sucrose has been supplied as carbohydrate, glucose of sufficient purity being no longer available. The sucrose has been purified by crystallization out of methylalcohol. STEINBERG

who in his experiments generally supplied sucrose, could not note any difference in effect between both sugars (27). In my experiments sucrose induced a somewhat slower growth so that the differences in sporangial development were later to occur than in the presence of glucose.

In Exp. 3–5 a range of smaller differences between the initial degrees was put up. In the unacidified medium (Exp. 5) hardly any difference existed between the production of sporangia in the (— Mn) cultures and those supplied with Mn.

Column 7 and 8 of Table I show that when the yields in weight are compared in the acidified media increase in the supply of Mn does not correlate with an increase in yield, on the contrary the (— Mn) cultures, where development of sporangia is completely suppressed, are highest in yield. This phenomenon will be discussed more in detail later.

In the non-acidified series (Exp. 5) where the (— Mn) cultures had developed sporangia, the addition of 0.005 γ Mn results in a slightly higher yield. Again the sensitiveness for traces of Mn is such and differences noted are so slight that a weighing of the fungus mats would not supply any information as to their provision with manganese.

In Exp. 4 and 5 the effect on the yield between a medium provided with KNO_3 Merck pro analyse and one with recrystallized pro analyse can be compared. In the unacidified medium the sporangial growth was profuse, the yield, however, in the medium provided with the recrystallized salt lower than in the other. It might be conceived that the purification of the solution had been such that a deficiency of some other oligopleront had occurred. This, however, has not been further investigated. The acidified medium furnished no lower yield and its sensitiveness for traces of Mn was the same as in case of the usual brand. The KNO_3 Merck pro analyse may be deemed free from any contamination with Mn.

When the results of STEINBERG (23–27) are compared with the above a marked difference may be noted. The yield of his — non-acidified — cultures remains always significantly lower in the absence of Mn than in its presence. He uses, however, $NaNO_3$ in stead of KNO_3 as a source of nitrogen. So I put up a range with $NaNO_3$ in order to ascertain whether this might explain the discrepancy. In Table II the results are registrated.

In Exp. 1 on the non-acidified medium the (— Mn) solution gives rise to a fair amount of sporangia and a dose as low as 0.1 γ Mn induces their optimal development. The weight of the yield in the absence of Mn equals that of the others. In Exp. 2 the effect of smaller doses of Mn is compared. The yield of the (— Mn) cultures is distinctly higher than in the presence of 0.01 γ Mn and the sporangial development is slighter. When Exp. 2 was repeated with sucrose as source of carbohydrate, similar results were arrived at.

Table II. Medium with $NaNO_3$ and glucose.

Exp.	Per culture		pH		Sporangia	Yield in g	Yield as % of (— Mn) cultures
	ml H_3PO_4	γ Mn	initial	final			
			Non-acidified medium				
1	0	0	6.6	5.2	++	0.662	100
	0	0.005	6.6	5.2	+++	0.672	102
	0	0.01 *)	6.6	5.2	++++	0.695	105
	0	0.2 *)	6.6	5.2	++++	0.665	101
2	0	0	6.6	5.5	+	0.77	100
	0	0.005	6.6	5.5	+±	0.71	92
	0	0.01	6.6	5.2	++	0.70	91
	0	0.025	6.6	5.2	++	0.75	97
			Acidified medium				
3	2.5	0	1.6	1.6	—	0.256	100
	2.5	0.05	1.6	3.6	+	0.68	266
	2.5	0.1	1.6	3.6	+	0.77	301
	2.5	0.2	1.6	3.0	++	0.74	289
	2.5	0.3	1.6	2.8	+++	0.76	297
	2.5	0.4	1.6	2.5	+++±	0.75	293

*) not in duplo.

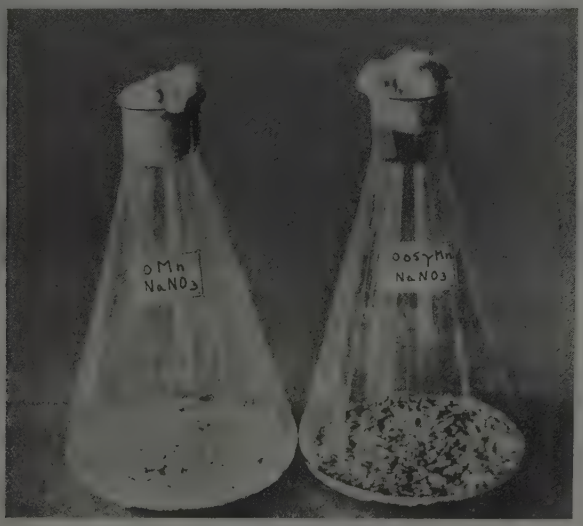

Fig. 3. $NaNO_3$ as source of nitrogen.
flask 1: — Mn + H_3PO_4 (2.5 ml 3.21 n)
flask 2: 0.05γ Mn + H_3PO_4 (2.5 ml 3.21 n)

When acidified by 2.5 ml H_3PO_4 the final pH attained by the medium was 1.6, and 0.05 γ Mn induced a distinct development of sporangia (Fig. 3).

A gift of 0.4 γ resulted in the nearly optimal development. The yield of mycelium in the absence of Mn was very materially lower than in its presence; 0.1 γ Mn brought the yield on the level of higher gifts.

In another experiment the effect on visual growth of increasing amounts of H_3PO_4 in the absence of Mn is compared. 1.5 ml induced a final pH of 3.2, 2 ml of 2.5 and 2.5 ml of 2.2. Fig. 4 shows clearly the difference in development of sporangia; pH 3.2 allows of an optimal development, whilst 2.2 inhibits the growth of sporangia completely. So on this medium *Aspergillus niger* is very sensitive for slight differences in pH.

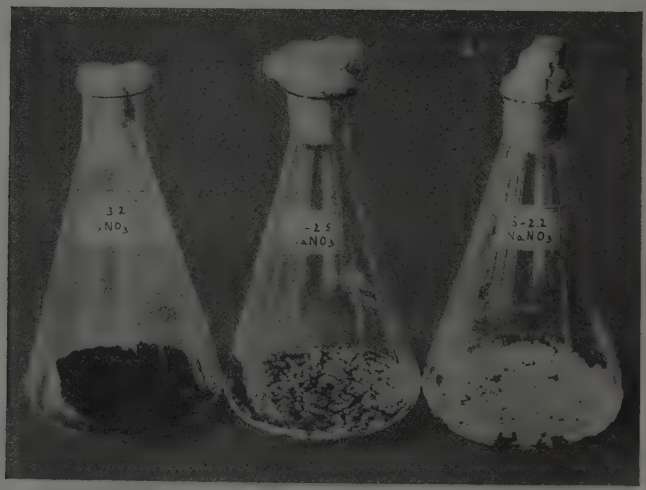

Fig. 4. $NaNO_3$ as source of nitrogen.
flask 1: — Mn + 1.5 ml H_3PO_4
flask 2: — Mn + 2.0 ml H_3PO_4
flask 3: — Mn + 2.5 ml H_3PO_4

In order to study the influence of lower amounts of Mn on the yield, Exp. 3 of Table II was repeated. Pure glucose being no longer available it has been substituted by sucrose.

Table III. Medium $NaNO_3$ and sucrose.

Exp.	Per culture		pH		Spo-rangia	Yield in g	Yield as % of (— Mn) cultures
	ml H_3PO_4	γ Mn	initial	final			
1	2.5	0	1.6	1.6	—	0.115	100
	2.5	0.005	1.6	1.6	—	0.34	296
	2.5	1	1.6	1.6	+	0.774	673
	2	0	1.6	1.6	—	0.315	100
	2	0.005	1.6	1.6	—	0.46	146
	2	1	1.6	1.6	+++	0.80	254
2	2.5	0	1.6	1.6	—	0.44	100
	2.5	0.005	1.6	1.6	—	0.625	142
	2.5	0.01	1.6	1.6	—	0.57	130
	2	0	1.6	2.3	—	0.772	100
	2	0.005	1.6	2.3	—	0.765	99
	2	0.01	1.6	2.3	—	0.760	98
	2	0.1	1.6	2.3	—	0.775	100
3	2.5	0	1.6	2.2	—	0.76	100
	2.5	0.005	1.6	2.2	—	0.83	109
	2.5	0.01	1.6	2.2	—	0.83	109
	2	0	1.6	3.5	—	0.89	100
	2	0.005	1.6	3.5	—	0.87	98
	2	0.01	1.6	3.5	—	0.87	98

Table III shows that as long as the final pH remains very low, the marked increase in weight induced by a dose as low as 0.005 γ Mn may be noted. When, however, the final pH is somewhat higher (Exp. 3) this influence is much slighter. When 2 ml H_3PO_4 has been supplied the yield of the (— Mn) cultures equalled that of the others.

b). Ammonium nitrate as a source of nitrogen.

As a difficulty met with in both former media lay in the fact that they had to be acidified strongly in order to maintain a high H-ion concentration up till the end, this involving the risk of inhibition of the initial development, NH_4NO_3 was tried as a source of nitrogen. It was indeed to be expected that the trend for alkalinisation would be weaker in such a medium. In fact when 3.1 g NH_4NO_3 per liter had been supplied to the medium, 1 ml H_3PO_4 appeared sufficient for keeping the pH below 1.9.

Table IV shows that 0.8 γ Mn is needed for a development of sporangia very near the optimal. So the acidified NH_4NO_3 medium is less sensitive in its response for minute traces of Mn than both the former solutions and a larger number of visually to be distinguished degrees can be realised. As far as this goes it might prove more suitable for practical purposes. Fig. 5 represents such a graded scale.

Table IV. Acidified medium with NH_4NO_3 and glucose.

Per culture		pH		Sporangia	Yield in g	Yield as % of (— Mn) cultures
ml H_3PO_4	γ Mn	initial	final			
1	0	1.6	1.9	—	0.585	100
1	0.05	1.6	1.6	±	0.58	99
1	0.1	1.6	1.9	±		
1	0.2	1.6	1.9	+ ±		
1	0.4	1.6	1.9	+ + +		
1	0.6	1.6	1.9	+ + +		
1	0.8	1.6	1.9	+ + + ±		
1	1	1.6	1.9	+ + + +	0.64	109

In order to study the effect on the yield in weight of small doses of Mn in an acidified medium the experiment of Table V was put up.

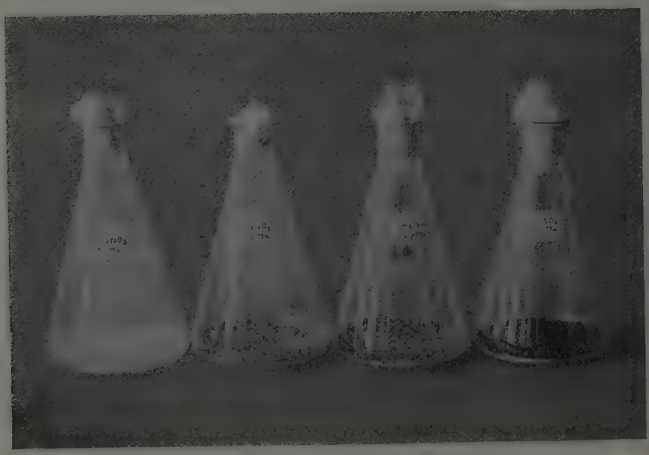

Fig. 5. NH_4NO_3 as source of nitrogen; 1 ml H_3PO_4 added. flask 1: — Mn; flask 2: + 0.1 γ Mn; flask 3: + 0.2 γ Mn; flask 4: + 0.5 γ Mn.

Table V. Acidified medium with NH_4NO_3 and sucrose.

Per culture		pH		Sporangia	Yield in g	Yield as % of (— Mn) cultures
ml H_3PO_4	γ Mn	initial	final			
1.5	0	1.6	1.6	—	0.97	100
1.5	0.005	1.6	1.6	—	0.765	79
1.5	0.01	1.6	1.6	—	0.885	91

In contrast with the results noted in the $NaNO_3$ medium, here the omission of Mn in a medium with a high final acidity does not result in a low yield. Apparently it is not the acidity of the medium as such which involves a weak growth of mycelium in the absence of Mn.

Table VI. Medium with NH_4NO_3 and glucose.

Per culture		pH		Sporangia	Yield in g	Yield as % of 1 ml H_3PO_4 culture
ml H_3PO_4	γ Mn	initial	final			
1	0	1.6	1.6	—	0.585	100
0.5	0	1.9	2.5	++++	0.59	101
0	0	6.7	3.8	++++	0.50	85
1	0.05	1.6	1.6	±	0.58	100
0.5	0.05	1.9	2.5	++++	0.59	102
0	0.05	6.7	3.8	++++	0.51	88

Table VI learns that the medium with NH_4NO_3 as well is very sensitive for slight raisings of pH. As may be taken from Table VI the addition of 0.5 ml H_3PO_4 will result in a final pH of 2.5; under these conditions the (— Mn) cultures will produce optimal growth of sporangia, the pH rendering the presence of Mn superfluous.

c). Ammonium as a source of nitrogen.

Finally the action of ammonium as only source of nitrogen was tested. Most of these experiments have been carried out with NH_4Cl, a lesser number with $(NH_4)_2SO_4$; the results arrived at were of a same nature.

When no Mn has been intentionally added a coalescent white felt is formed and any development of sporangia is suppressed. The acidification occurs at a quick rate and the final pH is 1.6. The addition of small doses of Mn induces the growth of sporangia; their development, however, is always less dense than when nitrate has been supplied. In a fairly wide range (0.1 γ up till 0.7 γ, Fig. 6) the development may remain sparse and no clear-cut differences may be met with. And even the optimal development will not produce a completely covered black mat: the sporangia will occur in evenly scattered black tufts with small uncovered regions between. This has to be ascribed to the acidity of the medium involved by the development of the fungus. In the acidified nitrate medium a similar phenomenon has been stated to occur.

The amounts of manganese inducing equal degrees of sporangial development varied considerably in the various experimental sets, duplo's however corresponding closely. Fig. 6 in which the curves connect the amounts of manganese inducing an equal sporangial development in various sets, illustrates this clearly. It might be conceived that this erratic behaviour was caused by

variety in amounts of the inoculum, the suspensions used being of varying density. It was attempted to answer this experimentally. In media containing 0.3 γ and 0.5 γ Mn per culture inocula consisting out of 1, 3 or 5 drops did not, however, influence the resulting degree of development. Thus the variety in the density of sporangia in equal concentrations of manganese may not be ascribed to a difference in the amount of spores in the inoculum. Still a difference in rate or measure of acidification of the medium will probably be cause of the variety. As long as this discrepancy in results may not be prevented, an unbuffered medium with ammonium as only source of nitrogen is quite unsuitable as a visual manganese standard.

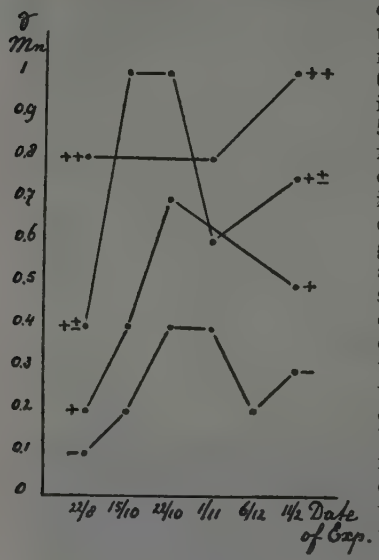

Fig. 6.
Amounts of manganese inducing equal sporangial development in various experimental sets.

The weight of the yields induced by the various amounts of Mn has been ascertained. Table VII offers an example.

Table VII. Medium with NH_4Cl and glucose.

Per culture	pH		Sporangia	Yield in g	Yield as % of (— Mn) culture
γ Mn	initial	final			
0	6.6	1.9	—	0.564	100
0.1	6.6	1.9	—	0.51	90
0.25	6.6	1.9	— (±)	0.59	105
0.5	6.6	1.9	+ ±	0.61	108
0.75	6.6	1.9	+ +	0.555	98
1	6.6	1.9	+ +	0.58	103
1.25	6.6	1.9	+ +	0.595	106
1.5	6.6	1.9	+ +	0.545	97

There is no definite trend in weight of the yield induced by increasing amounts of manganese. When the yield for 0.1 γ Mn is expressed as % of the yield in the absence of Mn, four different experimental sets offer as results 105%, 99%, 95% and 90%. So the yield in the absence of Mn may easily be higher than in its presence and an ascertainment by weight of the yields will not allow of any conclusions as to their content of Mn.

The effect was tried of a supply of $CaCO_3$ to the medium as a buffer, which as has been stated already, will allow of a profuse development of sporangia in the absence of manganese. For this purpose rigidly pure $CaCO_3$ had been prepared. The optimal dose of $CaCO_3$ per culture had to be ascertained. Table VIII presents the results.

Table VIII. Medium with NH_4Cl and glucose.

Exp.	Per culture		pH		Spo-rangia	Yield in g	Yield as % of	
	g $CaCO_3$	γ Mn	initial	final			(–$CaCO_3$) culture	(— Mn) culture
1	0	0	6.6	1.6	—	0.6	100	
	0.062	0	6.6	1.9	+±	0.72	120	
	0.125	0	6.6	3.8	++	1.15	192	
	0.250	10	6.6	6.1	++	0.94	157	
	0	10	6.6	1.6	+++	0.515	100	
	0.062	10	6.6	2.1	+++	0.6	117	
	0.125	10	6.6	4.1	++++	1.225	238	
	0.250	10	6.6	6.1	++++	0.945	183	
2	0.125	0	6.6	5.5	+++±	0.740		100
	0.125	0.05	6.6	6.1	++++	0.785		106
	0.125	0.10	6.6	5.5	++++	0.780		105
	0.125	0.20	6.6	5.5	++++	0.780		105

It shows clearly that in the absence as well as in the presence of Mn a dose of 0.125 g $CaCO_3$ produced the highest yields, respectively 192% and 238% of the amount in the absence of $CaCO_3$. A dose of 0.25 g is apparently too high; the final pH of 6.1 will presumably induce less favourable growing conditions for *Aspergillus niger*, be it that some nutritional substance has been precipitated. In the absence of Mn the cultures with a final pH of 1.6 and 2.1 have produced a higher yield in weight than the corresponding cultures which had been supplied with 10 γ Mn. The latter cultures had developed sporangia more profusely which may have influenced unfavourably the amount of mycelium synthetised. The yield of the cultures with the larger amounts of $CaCO_3$ was markedly higher than any yields arrived at in the nitrate series.

The experiments with an ammonium salt as only source of nitrogen indicate, that the ascertainment by weight of the yields for increasing amounts of Mn cannot furnish a manganese standard.

3. Discussion.

The experiments have furnished evidence that in a medium of very low pH manganese is essential for the development of sporangia. Is it, however, essential as well for the development of mycelium?

Table IX.

Culture medium	— Mn		With highest sporangia development					With 0.005 γ Mn			
	Final pH	Spor.	Final pH	Spor.	γ Mn	Yield in g	Yield as % of (— Mn) culture	Final pH	Spor.	Yield in g	Yield as % of (— Mn) culture
KNO_3, glucose	1.6	—	2.5	++++	0.8	0.670	88		—		
idem	2.2	—	2.3	++++	1.0	0.642	74		—		
KNO_3, sucrose	2.2	—						2.5		0.83	98
KNO_3 recryst. sucrose	2.5	—						2.2		0.77	94
$NaNO_3$, glucose	1.6	—	2.5	+++±	0.4	0.75	293				
$NaNO_3$, sucrose	1.6	—	1.6	+	1.0	0.774	673	1.6	—	0.34	296
idem	1.6	—	1.6	+++	1.0	0.80	254	1.6	—	0.46	146
idem	1.6	—						1.6	—	0.625	142
idem	2.3	—						2.3	—	0.765	99
idem	2.2	—						2.2	—	0.83	109
idem	3.5	—						3.5	—	0.87	98
NH_4NO_3, glucose	1.9	—	1.9	+++±	1	0.64	109				
idem	1.6	—	1.6	±	0.05	0.58	99				
NH_4NO_3, sucrose	1.6	—						1.6	—	0.765	79

In Table IX have been brought together the data bearing on this question of the experimental sets in which a low pH has involved the suppression of sporangia in the absence of Mn. The yields of the (— Mn) cultures are compared with those of cultures which had attained the best sporangia development in the particular set and (or) with the yields of 0.005 γ Mn cultures. In the KNO_3 sets the yields of the (— Mn) cultures without sporangia are higher than those of cultures where the supply of manganese had involved development of sporangia. It may be conceived that as a result of the formation of sporangia some nutritional substance other than Mn might come in the minimum; this would involve an inhibition of the growth of mycelium at an earlier stage than in the absence of manganese. On the NH_4NO_3 medium no definite trend in the relation of the yields in absence or presence of Mn could be ascertained, neither could this be stated for NH_4Cl.

On the medium with $NaNO_3$, however, the yield in the absence of Mn is very markedly lower than in its presence. As long as a very low pH prevails under strongly acid conditions manganese is here apparently essential for the development of mycelia as well as of sporangia. Like acid conditions, however, do not involve a similar inhibition of mycelial development when other sources of nitrogen have been supplied to the medium. Thus the marked feature of $NaNO_3$ cultures may not be wholly ascribed to the influence of a low pH, although it is a condition *sine qua non* for its occurrence. As has been stated already the deviating results with the $NaNO_3$ medium apparently neither may be ascribed to a greater freedom from last traces of manganese of this salt. So the discrepancy has to be ascribed to a difference in metabolism on the acidified $NaNO_3$ medium.

Table X contains data of less acid sets where sporangia developed in the absence of manganese. Under those conditions no definite trend in the relation between mycelial yield in the absence and presence of Mn can be ascertained and under these conditions $NaNO_3$ induces no deviation in behaviour.

Might it, however, be possible to explain the occurrence of deficiency symptoms in either the sporangial or the mycelial development in highly acid media by an action of the medium itself on the availability of manganese? In a more acid medium manganese might on the contrary be expected to exist in a more soluble form and thus manganese deficiency would be less apt to occur. Thus the case of the difference in action between media of lower and higher pH has in fact not to be looked for in an action in the medium but in an action of the fungus.

So the intake of manganese by *Aspergillus niger* under varying conditions has been studied by means of estimation of the content of Mn in the yield. In this end the culture media had been supplied with higher gifts of Mn, 150 γ or 200 γ per culture. The fungous mats were removed out of the culture flasks, rinsed in water, dried at 105° C. and weighed. The amount of manganese was

Table X.

Culture medium	← Mn		With highest sporangial development				
	Final pH	Spor.	Final pH	Spor.	γ Mn	Yield in g	Yield as % of (— Mn) culture
KNO_3, sucrose	5.2	++++	5.2	++++	1	0.915	116
KNO_3 recryst. sucrose	5.2	++++	5.2	++++	1	0.787	104
$NaNO_3$, glucose	5.2	++	5.2	+++	0.01	0.695	105
idem	5.5	±	5.2	++	0.01	0.700	91
NH_4NO_3, glucose	2.5	++++	2.5	++++	0.05	0.590	101
idem	3.8	++++	3.8	++++	0.05	0.510	88
NH_4Cl, glucose	3.8	++	4.1	++++	10	1.225	110
idem	6.1	+++±	6.1	++++	0.05	0.785	106

Table XI.

Medium	γ Mn	Number of cultures	pH		Yield in g per culture	γ Mn in 0.5 g
			initial	final		
NH_4Cl+0.25 g $CaCO_3$	200	1	6.6	5.8	0.83	30
idem	200	1	6.6	5.8	0.79	20
NH_4Cl	200	1	6.6	1.6	0.56	15
idem	200	1	6.6	1.6	0.57	20
NH_4NO_3	200	1	6.4	3.2	0.73	22.5
idem	200	1	6.4	2.9	0.76	40
idem	200	1	6.4	3.7	0.66	22.5
idem	200	3	6.4	3.4	0.62	40
NH_4NO_3+1 ml H_3PO_4	200	1	1.6	1.9	0.66	15
idem	200	1	1.6	1.9	0.69	17.5
idem	200	1	1.6	1.9	0.65	15
idem	200	3	1.6	1.6	0.61	5
KNO_3	200	3	6.4	5.2	0.74	30
KNO_3+2 ml H_3PO_4	200	3	1.6	2.8	0.69	10
$NaNO_3$	150	3	6.4	6.7	0.67	20
$NaNO_3$+2 ml H_3PO_4	150	3	1.6	2.8	0.77	10

estimated according to the method of MARSHALL (12), amended by STEENBJERG (22).

A sample of 0.5 g dry weight is transferred to a Kjeldahl flask, 5 ml HNO_3 and 6 ml H_2SO_4 are added and the flask is heated on a low flame, later on a higher until the liquid is colourless. After addition of 15 ml distilled water the solution is neutralized with 1 ml 6 n NaOH, and 1.25 ml H_2SO_4 is added in excess. The solution is filtered of and the filter washed with boiling water. 1 ml 6 n HNO_3 and 2 ml 0.1 n $AgNO_3$ are added. After the solution has been supplied with a spatula ammonium persulphate it is carefully heated (on the verge of boiling) up till the appearance of the pink shade of permanganate. After one more minute near boiling temperature it is cooled down and transferred to a colorimeter glass of 100 ml. The colorimeter scale ranges from 5—65 γ Mn and is sensitive for differences of 5 γ. In order to render the standard solution more permanent it is provided with some potassium perjodate.

Table XI presents the results. The relatively high dose of 200 γ Mn per culture induced no high intake, 40 γ Mn/0.5 g being the maximum. Differences in intake are slight. The strongly acid cultures show a somewhat lower content of the yield than the cultures with a higher final pH. So it is not quite out of question, that the fact that sporangia will develop in media of lesser acidity in the absence of intentionally added manganese, might be caused by a better intake under those conditions of still prevailing traces of manganese. As such traces cannot be detected chemically, direct evidence cannot be gained.

As has been stated already the sporangial development in an unbuffered ammonium salt medium responds less quickly on traces of Mn than in a nitrate medium. The data in table XI prove that this may not be explained by a weaker intake of Mn in the former medium. The difference must be due to a difference between the metabolic processes of the fungus in both media.

It was further tested whether the intake of manganese would correspond with an increase in supply of the medium. As such a buffered NH_4Cl medium was used.

Table XII.

γ Mn	Number of cultures	pH		Yield in g per culture	γ Mn in 0.5 g
		initial	final		
10	2	6.6	5.8	0.685	5
20	2	6.6	5.8	0.825	5
40	1	6.6	5.8	0.750	10
40	1	6.6	6.1	0.580	15
80	1	6.6	6.4	0.740	15
80	1	6.6	6.1	0.740	10

Table XII shows that no such correlation exists. Estimation of the content of manganese of the yield in mycelium would not

provide any information about the amount of manganese supplied to the fungus,

The results arrived at indicate, that manganese plays quite another part in the metabolism of *Aspergillus niger* than molybdenum, although for oats results of BURSTRÖM and LUNDEGAARDH might seem to point at some similar action. Whilst molybdenum is merely essential when nitrate is the only source of nitrogen, in case of manganese the difference between nitrate and ammonium plays no part. The chief deficiency symptom, the suppression of sporangia, however, depends on the pH of the medium and only occurs at a low pH. At a higher pH sporangia will develop undependently of the prevailing source of nitrogen. Under such conditions all essential synthetic processes are apparently possible in the absence of manganese. Merely a deeper biochemical investigation of the metabolism of *Aspergillus niger* might furnish some explanation for the difference in need of manganese under various conditions.

My results need comparison with those arrived at by other investigators.

BERTRAND was able to induce differences in yield by the supply of 0.005 γ Mn when compared with (— Mn) cultures. When $NaNO_3$ had been supplied as source of nitrogen I arrived at the same result. Apparently the simple method of purification by adsorption served as well for the removal of the last traces of manganese as the more elaborate means applied by BERTRAND.

Between the results of STEINBERG (25, 26) and mine some discrepancies are manifest, although I have meant to follow his methods exactly. Without any previous acidification of the culture media the final pH of his various nitrate solutions ranges from 1.58—2.11 and is thus much lower than I could ascertain in corresponding experiments. This may explain, however, that in the not intentionally acidified nitrate media STEINBERG often notes a complete suppression of sporangia in the absence of manganese. In some experiments, however, sporangia did occur; for instance a culture with $NaNO_3$ and a final pH of 1.89 and another with NH_4NO_3 and a final pH of 1.67 produced sporangia at about the level designed by me as +. Moreover he could note in the absence of Mn a constantly smaller yield of mycelium in its presence: f.i. a culture with NH_4NO_3 produced 68% of the yield of one supplied with Mn. In my experiments I could merely note in a highly acidified $NaNO_3$ medium a lower yield of mycelium in the absence of Mn than in its presence. I am unable to indicate the cause of these discrepancies.

In a paper of BORTELS in 1927 (6) on the significance of iron, zinc and copper for *Aspergillus niger*, he notes that in acid media growth is slight and formation of spores is suppressed; when such a culture solution is rendered more alkaline sporangia will develop abundantly. He was the first to introduce the purification of the medium by adsorption on coal and did not intentionally supply

any manganese. Although he looks for an explanation in a direct action of H′ in the presence of much Zn, it seems to me very probable that a manganese deficiency had come into play there.

Finally a graded scale for manganese has to be considered as to its suitability for a practical soil test. When merely the response to increasing amounts of Mn is kept in view, the acidified scale with NH_4NO_3 as source of nitrogen might seem promising. For carrying out a soil test a soil sample of known weight has to be transferred to the culture medium. In any alkaline soil, however, some insoluble manganese may be expected to occur, which at the low pH of the medium will be converted into a manganous salt and thus will go into solution. Any manganese detected by means of the development of sporangia would prove nothing about the actual occurrence of plant available manganese in the soil sample. Alkaline soils, thus soils in need of estimation of their available manganese, might only be tested by means of an about neutral buffered solution. In such a medium, however, manganese appears to be unessential for the development of sporangia.

Neither would an estimation of the content of Mn in the yield of mycelium furnish any information about the amount supplied.

For practical purpose as a test for plant available manganese the *Aspergillus* method, such I could contrive to make it up, appears to be unsuitable.

Summary.

1. The response of *Aspergillus niger* to the presence of manganese in the nutrient solution has been ascertained.
2. In an initially acidified medium which kept up its acidity or in an unbuffered neutral medium which grew strongly acid, manganese appeared essential for sporangial development.
3. In suitably acidified solutions KNO_3 as source of nitrogen induced slight sporangial development in the presence of 0.05 γ Mn, $NaNO_3$ of 0.025 γ Mn and NH_4NO_3 of 0.05 γ Mn. In the acidified NH_4NO_3 medium the greatest number of visually to be distinguished degrees could be ascertained.
4. In acidified solutions with KNO_3 the yield of mycelium was higher in the absence of manganese than in cultures which in its presence had developed sporangia. Presumably the development of sporangia inhibits further development of mycelium.
5. The weight in yield of mycelium in an acidified $NaNO_3$ solution in the absence of manganese was very significantly lower than in the presence of 0.005 γ Mn.
6. In non-acidified nitrate solutions sporangia developed in the absence of manganese. No definite trend in the yield of mycelium could be ascertained.
7. In unbuffered solutions with ammonium salts no sporangia developed in the absence of manganese. The response on

various amounts of manganese was erratic. No definite trend in mycelial development could be ascertained.

8. Solutions with an ammonium salt suitably buffered by $CaCO_3$ produced sporangia in the absence of manganese. They gave the highest yields in weight.
9. The intake of manganese by the fungus on various media offers slight differences. The intake is slightly higher in weakly acid than in strongly acid solutions.
10. The fact that manganese is only essential in a highly acid medium may not be ascribed to an action of the medium as such and must be caused by differences in the metabolism of *Aspergillus niger*.
11. As *Aspergillus niger* is sensitive for traces of manganese only under highly acid conditions an *Aspergillus* standard will not be suitable for soil tests.

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(From „Bedrijfsgroep Zuivel- en Melkproductenindustrie en Melkinrichtingswezen", La Haye).

RAPPORT ENTRE LA TENEUR EN HUMIDITÉ DU LAIT SEC MAIGRE (PROVENANT DE LAIT ÉCRÉMÉ) ET LE DÉVELOPPEMENT D'UNE FLORE MICROBIENNE DANS CE LAIT

par

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(Reçu le 1 août 1944).

1. Introduction.

La bibliographie de la question nous apprend, que dans presque tous les laits secs, ou poudres de lait, le nombre de germes est bas et que pendant la conservation le nombre de micro-organismes augmente à peine ou point du tout, mais qu'au contraire il diminue le plus souvent. En général l'état de siccité de ces laits est considéré comme l'obstacle au développement d'une flore microbienne.

Les laits secs, surtout celui qui est obtenu par pulvérisation, possèdent une très grande hygroscopicité; dans un emballage non hermétique ils reprennent vite de l'humidité. Supplee (3) a établi l'équilibre des teneurs en humidité du lait sec en le conservant dans des milieux exposés à une tension de vapeur relative variant de 10 à 80%. Il a trouvé, le fait remarquable, que dans une humidité relative dépassant 40%, la reprise d'eau s'élevait en peu de temps à un maximum et qu'ensuite la teneur en humidité de la poudre rebaissait graduellement jusqu'à un certain point d'équilibre dépendant naturellement de l'humidité de l'air ambiant. Quand la teneur en humidité relative du milieu était au-dessus de 80%, la poudre commençait à moisir sous peu.

Supplee ne dit pas s'il a fait des recherches ultérieures sur la moisissure en rapport avec l'humidité de la poudre et l'air ambiant. Tamsma (4) a établi l'équilibre des teneurs en humidité d'un échantillon de lait sec sous tension de vapeur relative de 20 à 50%. Ses résultats sont essentiellement conformes à ceux de Supplee. Que Tamsma diffère environ de 1% avec Supplee dans les points d'équilibres des teneurs en humidité, cela importe peu, les poudres différant de composition par suite de leur fabrication et de leur provenance différentes.

Or, il était intéressant de chercher à suivre exactement quel est le rapport entre la teneur en humidité de la poudre et les possibilités d'existence des bactéries, des levures et des moisissures à la température du laboratoire.

Pour expérimenter la chose nous avons conservé du lait sec

à divers degrés d'humidité relative. Les différents milieux ont été constitués dans des exsiccateurs à l'aide de solutions saturées de sel. Dans ces exsiccateurs nous avons placé une cuvette de verre contenant le lait sec; nous avons pris comme objet d'expérience un échantillon de poudre Hatmaker[1]) et un de poudre Krause[2]) sous des tensions de vapeur relatives de 30%, 75%, 80%, 85%, 90% et de 100%.

Nous avons employé les solutions de sel indiquées dans l'aperçu suivant:

Pour la tension de vapeur relative de 30% solution saturée de $CaCl_2$
" " " " " " " 75% " " " NaCl
" " " " " " " 80% " " " $(NH_4)_2SO_4$
" " " " " " " 90% " " " KCl
" " " " " " " 100% eau

2. Conservation du lait sec écrémé (maigre) dans des milieux exposés à différentes tensions de vapeur relative.

Dans notre première série d'essais de conservation nous avons conservé la poudre en boîtes de Petri sans couvercle et avons gardé les exsiccateurs à la température du laboratoire sans les protéger contre la lumière du jour. A un moment donné nous avons pris un échantillon dans la couche supérieure et celui-ci a fait l'objet de recherches sur le nombre de germes et la teneur en humidité. La teneur en humidité a été déterminée par dessiccation, jusqu'à un poids constant, dans une étuve chauffée à 103° C., d'une quantité déterminée de poudre dans une cuvette de porcelaine. Le nombre total de germes a été déterminé sur des plaques d'autolysat de levure et de peptone. Après inoculation les plaques ont été incubés pendant quatre jours à 25° C.

Le résultat sous les différentes tensions de vapeur est mentionné dans les 2 tableaux suivants: le premier se rapporte à la poudre Krause et le deuxième à la poudre Hatmaker. Dans le cours de notre rapport nous parlons toujours, pour plus de simplicité, de lait sec (ou poudre) Hatmaker et de lait sec (ou poudre) Krause.

Résultats des recherches.

En examinant les résultats obtenus on remarque tout d'abord qu'il y a bien quelquefois des constatations qui paraissent un peu invraisemblables si on les compare avec l'ensemble.

Les fluctuations de la teneur en humidité trouvent probablement leur cause dans ce que, pour l'analyse, on a pris la couche supérieure et qu' après on y a mélangé la poudre restante. Il est

[1]) Le lait sec Hatmaker s'obtient en faisant passer le lait en lamelles entre deux cylindres tengents, chauffés à une certaine température. La dessiccation est instantanée.

[2]) Le lait sec Krause est obtenu en pulvérisant le lait en gouttelettes et en l'envoyant dans une chambre chauffée par un courant d'air sec et chaud.

Tableau I.

durée de conservation en jours	humidité relative de: 100% nombre de germes	100% teneur en humidité %	90% nombre de germes	90% teneur en humidité %	85% nombre de germes	85% teneur en humidité %	80% nombre de germes	80% teneur en humidité %	75% nombre de germes	75% teneur en humidité %	30% nombre de germes	30% teneur en humidité %
0	15000	4.0	16000	4.0	11500	4.0	11500	4.0	18000	4.8	18000	4.8
4	—	—	—	—	12000	—	7000	—	—	—	—	—
8	2400	8.5	2000	9.4	8000	7.2	4000	6.7	—	—	—	—
13	1400	9.6	1300	10.6	2200	—	3000	—	1300	—	2500	—
17	1600	8.0	1500	8.4	1300	8.7	900	10.0	—	—	—	—
21	1100	10.2	1700	9.9	5000	9.8	1000	9.9	—	—	—	—
25	1300	9.9	1300	10.2	4000	9.4	4000	10.3	—	—	—	—
29	1000	10.3	1000	10.7	3000	—	1000	—	1400	—	2600	—
34	1100	13.1	1000	11.2	3000	9.5	1500	11.2	—	—	—	—
38	1000	13.9	1000	11.3	3000	10.5	1000	11.4	—	—	—	—
42	4000	14.1	3100	13.1	1500	10.9	1200	11.8	—	—	—	—
46	4000	13.0	3500	14.1	1 00	11.0	1500	11.9	1200	—	600	—
50	5700	16.5	5000	14.0	—	—	—	—	—	—	—	—
54	9500	—	21000	—	—	11.7	2500	12.0	—	—	—	—
58	moisie	18.4	moisie	15.9	1300	11.6	2100	11.8	700	—	300	—
63	—	—	—	—	—	12.6	2000	12.0	—	—	—	—
67	—	—	—	—	2000	12.1	2000	12.0	—	—	—	—
71	—	—	—	—	4700	12.3	15000	12.9	1700	—	600	—
75	—	—	—	—	—	12.4	moisie	12.9	—	—	—	—
83	—	—	—	—	3000	12.7	—	—	—	—	—	—
87	—	—	—	—	4300	14.2	—	—	1300	—	2800	—
91	—	—	—	—	1100	13.1	—	—	—	—	—	—
96	—	—	—	—	2300	—	—	—	—	—	—	—
100	—	—	—	—	15000	14.7	—	—	900	—	1700	—
104	—	—	—	—	moisie	13.8	—	—	—	—	—	—
114	—	—	—	—	—	—	—	—	4500	—	7500	—
128	—	—	—	—	—	—	—	—	1300	—	900	—
142	—	—	—	—	—	—	—	—	400	10.8	1500	6.0
156	—	—	—	—	—	—	—	—	1600	—	500	—
180	—	—	—	—	—	—	—	—	1600	12.8	—	—

Tableau II.

durée de conservation en jours	humidité relative de:											
	100%		90%		85%		80%		75%		30%	
	nombre de germes	teneur en humidité %	nombre de germes	teneur en humidité %	nombre de germes	teneur en humidité %	nombre de germes	teneur en humidité %	nombre de germes	teneur en humidité %	nombre de germes	teneur en humidité %
0	3000	5.1	2400	5.1	15000	4.5	15000	4.5	12000	5.3	12000	5.3
4	15000	9.1	2500	8.7	3000	7.8	—	8.8	—	—	—	—
8	16000	9.9	3200	10.0	4000	8.0	7000	10.8	—	—	—	—
13	4000	9.3	4500	11.8	26000	10.0	6000	12.2	3700	—	17000	—
17	19000	16.1	1000	—	15000	10.8	3000	11.9	—	—	—	—
21	30000	18.8	60000	16.8	6000	11.8	—	11.8	—	—	—	—
25	moisie	19.8	moisie	20.3	4500	12.3	—	11.8	—	—	—	—
29	—	—	—	—	4500	11.5	3500	11.0	1200	—	3500	—
34	—	—	—	—	—	12.4	—	11.2	—	—	—	—
38	—	—	—	—	5000	13.9	7000	13.1	—	—	—	—
42	—	—	—	—	2000	13.7	5000	12.9	500	—	1000	—
46	—	—	—	—	8000	14.4	1500	—	—	—	—	—
53	—	—	—	—	moisie	14.8	—	—	300	—	1200	—
71	—	—	—	—	—	—	1500	12.8	200	—	600	—
78	—	—	—	—	—	—	1500	13.6	—	—	—	—
82	—	—	—	—	—	—	3000	14.6	—	—	—	—
87	—	—	—	—	—	—	14000	13.3	1700	—	2600	—
92	—	—	—	—	—	—	1500	14.5	—	—	—	—
95	—	—	—	—	—	—	10000	13.5	—	—	—	—
100	—	—	—	—	—	—	moisie	13.5	500	—	1500	—
114	—	—	—	—	—	—	—	—	200	10.4	5000	6.1
128	—	—	—	—	—	—	—	—	300	—	200	—
142	—	—	—	—	—	—	—	—	1000	—	2600	—
170	—	—	—	—	—	—	—	—	3900	—	1100	—
184	—	—	—	—	—	—	—	—	1700	12.8	2400	6.7
212	—	—	—	—	—	—	—	—	1000	11.5	1000	6.1
231	—	—	—	—	—	—	—	—	600	11.1	2000	6.3
255	—	—	—	—	—	—	—	—	—	11.1	—	6.1

évident qu'ici „couche supérieure" a un sens très élastique et qu'une fois la couche était plus épaisse qu'une autre fois.

Les fluctuations du nombre de germes s'expliquent partiellement par la cause mentionnée ci-dessus. Toutefois l'inexactitude de la détermination même joue ici un grand rôle. En mélangeant de l'eau stérile avec de la poudre peu soluble on obtient difficilement un mélange homogène. Aussi c'est la plus grande difficulté que nous ayons eue en ce qui concerne la poudre Hatmaker. Malgré les inexactitudes mentionnées ci-dessus nous pouvons cependant tirer quelques conclusions de nos recherches.

Comme on pouvait s'y attendre, la poudre moisit d'autant plus vite que le degré d'humidité de l'air dans lequel on la conserve est plus élevé. L'essai fait à une teneur en humidité relative de 85% indiquée dans le tableau I fait une exception inexplicable à ce sujet. Les laits secs qui ont fait l'objet de nos recherches ne moisissaient pas même après un temps assez long de conservation à une humidité relative de l'air ambiant de 75% et de 30% quoique la teneur en humidité à 75% se fût assez élevée.

La teneur en humidité dans laquelle la moisissure se produit semble être dépendante du degré d'humidité de l'air dans lequel la poudre est conservée. Dans le tableau III, nous avons réuni les teneurs en humidité où commence la moisissure.

Tableau III.

Sorte de lait sec	teneur en humidité relative de l'air			
	100%	90%	85%	80%
Krause	18.4%	15.9%	13.8%	12.9%
Hatmaker	19.8%	20.3%	14.8%	13.5%

On y constate que cette teneur en humidité est d'autant plus haute, que la teneur en humidité relative de l'air dans lequel on conserve la poudre est plus élevée.

Le tableau III montre clairement, du moins en ce qui concerne les échantillons que nous avons examinés, que la poudre Hatmaker ne moisit qu'à un degré d'humidité plus élevé que la poudre Krause. En comparant les tableaux I et II il semble bien probable, que la poudre Hatmaker atteint ce degré d'humidité plus vite que la poudre Krause.

En conservant le lait sec, le nombre de germes baisse jusqu'à un certain niveau, assez bas, où il se maintient plus ou moins jusqu'à ce qu'une hausse se produise, à une certaine teneur en humidité. Nous pourrions appeler ce point d'humidité la „teneur en humidité critique".

Nous avons exprimé les données du tableau I d'après RICHTER-ALTSCHÄFFER (1) par des courbes correspondant aussi bien que possible aux constatations (voir fig. 1, 2, 3 et 4).

En ce qui concerne les résultats on remarquera ce qui suit. Les courbes indiquant le nombre de germes suivent toutes le même cours. Le premier tournant, terminant la première baisse

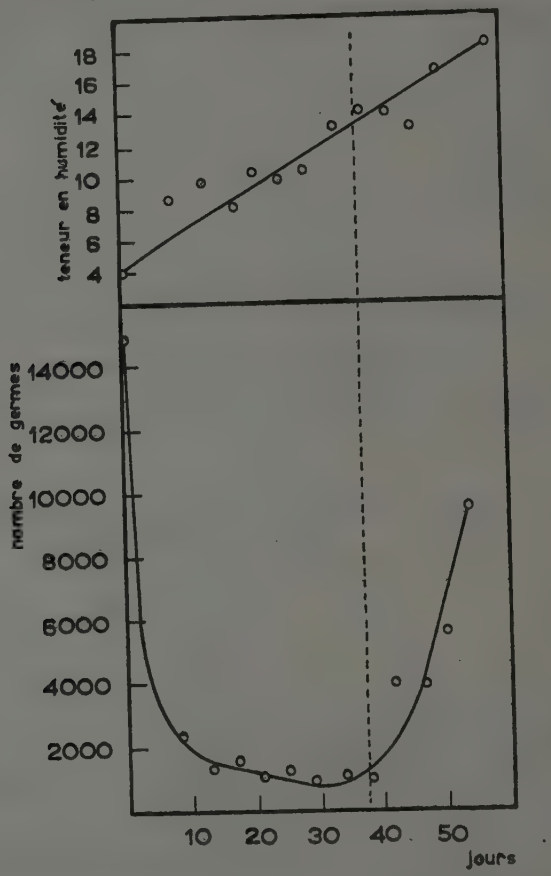

Fig. 1. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 100% d'humidité relative.

rapide du nombre de germes, est atteint: à 100% après 10 jours environ, à 90% après 10 jours environ, à 85% après 17 jours environ et à 80% après 17 jours environ.

Ensuite le nombre de germes reste assez constant jusqu'au moment où une hausse accentuée se produit tout à coup, à 100%

après 37 jours environ, à 90% après 38 jours environ, à 85% après 93 jours environ et à 80% après 68 jours environ.

Si nous considérons le degré d'humidité que la poudre a atteint au moment où la hausse du nombre de germes se produit, on

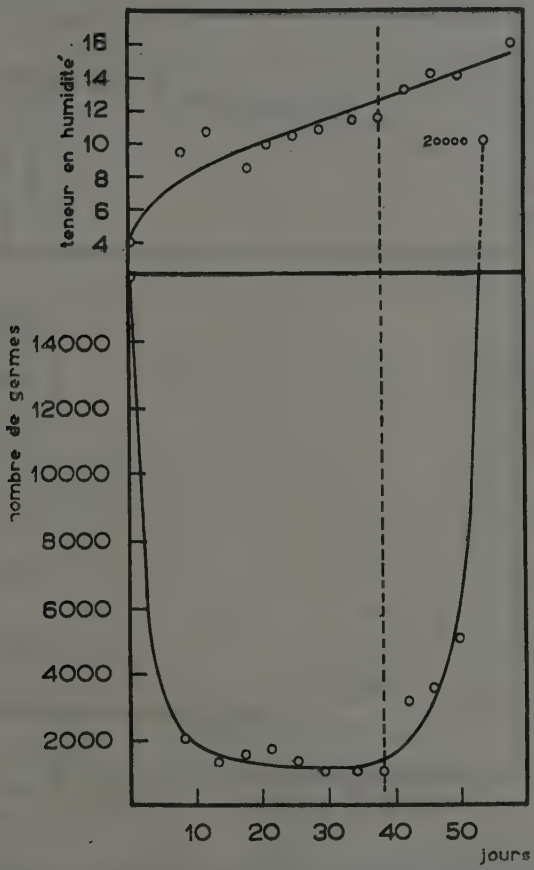

Fig. 2. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 90% d'humidité relative.

constate que ce degré d'humidité à 100% s'est élevé à 13.5%, à 90% s'est élevé à 12.5%, à 85% s'est élevé à 13.5% et à 80% s'est élevé à 12.5%.

Ces courbes nous montreraient donc que la hausse soudaine, indiquée ci-devant, du nombre de germes dans la poudre Krause,

se produit à une teneur en humidité déterminée, indépendamment des circonstances extérieures. Cela n'est pas nécessairement en contradiction avec ce que nous avons fait remarquer plus haut au sujet du degré d'humidité de la poudre en moisissure, puisque

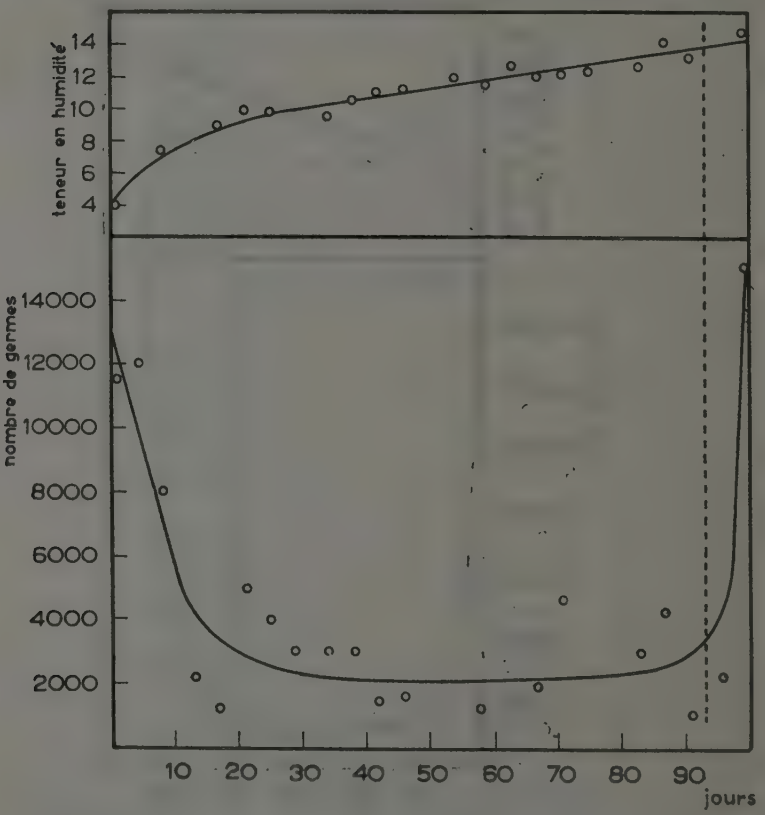

Fig. 3. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 85% d'humidité relative.

pour la production d'une moisissure visible il faut qu'une certaine quantité minimum de mycélium (avec conidies) soit formée. La rapidité du développement du mycélium dépendra certainement de la teneur en humidité relative de l'air ambiant, comme Schwartz et Kaesz (2) l'ont constaté dans des expérimentations concernant le développement des moisissures sur la viande.

3. Reprise de l'expérimentation avec des poudres Krause de fabrication différente.

Des expérimentations susmentionnées nous avons conclu que, très probablement, il existe pour le lait sec une „teneur en humidité critique", c'est à dire une teneur dans laquelle une flore microbienne

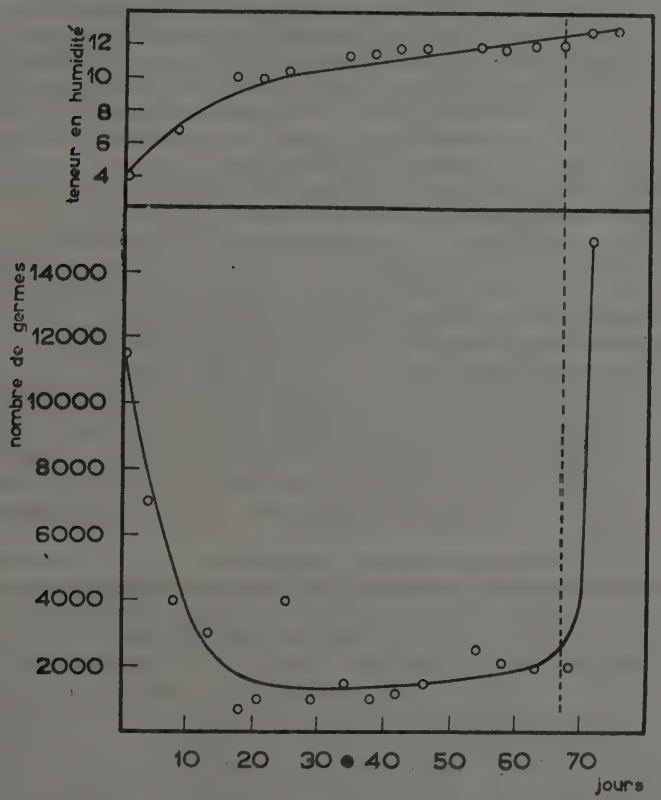

Fig. 4. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 80% d'humidité relative.

se développe très rapidement ce qui fait moisir la poudre en peu de temps. Au-dessous de cette teneur le nombre de germes reste constant ou diminue. Nous avons trouvé des indications qui portent à croire que la „teneur en humidité critique" d'une poudre est indépendante de la tension de vapeur relative sous laquelle cette poudre est conservée. Le degré d'humidité de la poudre où celle-ci a commencé à moisir visiblement s'est bien trouvé dépendant

de la tension de vapeur relative du milieu où on conservait la poudre.

Les recherches susdites n'ont été effectuées qu'avec une seule sorte de poudre Krause et de Hatmaker. Comme il n'était pas inimaginable que des poudres de fabriques différentes et produites à diverses époques donneraient des résultats différents, nous avons complété nos recherches par une série d'expériences avec cinq ou six poudres Krause provenant de diverses fabriques. De plus, il était intéressant d'établir l'équilibre de la teneur en humidité sous une certaine tension de vapeur relative. En conservant la poudre en une couche épaisse il s'écoulera beaucoup de temps avant qu'un équilibre ne se soit établi. C'est pourquoi nous avons procédé dans ces expériences en conservant la poudre en couches minces. Cela a, de plus, l'avantage que la reprise d'eau est plus rapide de sorte que la durée de l'expérience est plus courte.

Nous avons examiné les phénomènes qui se produisaient sous une tension de vapeur relative de 100% (au-dessus de l'eau), de 90% (au-dessus d'une solution saturée de Na_2SO_4), de 85% (au-dessus d'une solution saturée de KCl) et de 75% (au-dessus d'une solution saturée de NaCl). Dans les trois premières expériences susdites on a procédé comme suit. On a pesé environ 6 g de poudre dans une boîte de Petri. Cinq boîtes de Petri ont été placées l'une au-dessus de l'autre dans un exsiccateur où on avait mis respectivement de l'eau et la solution saturée de sel. Les exsiccateurs étaient placés dans l'obscurité et à la température du laboratoire. A des moments donnés tout le contenu d'une boîte a fait l'objet de recherches sur le degré d'humidité et le nombre de germes.

Les expérimentations sur la conservation à 75% d'humidité relative avaient un caractère un peu autre. Vu les résultats obtenus jusqu'ici, on ne s'attendait pas à ce que les poudres moisissent. C'est pourquoi nous avons seulement suivi le cours de la teneur en humidité tout en examinant surtout s'il s'établirait un état d'équilibre et comment se comporterait la teneur en humidité des diverses poudres dans cet état d'équilibre. La poudre était conservée dans l'obscurité et dans des cuvettes de porcelaine de 7 cm de diamètre. On en pesait exactement 2 grammes de sorte qu'ici, il s'agissait aussi d'une couche mince. La température de conservation était de 22° C. Les cuvettes étaient d'abord pesées tous les quatre jours, plus tard, à de plus grands intervalles. On calculait la teneur en humidité de la hausse d'équilibre trouvée. L'expérience a été faite en double.

Lorsque, au bout de deux mois, on constatait, ici encore, la présence de moisissure on a déterminé également le nombre de germes de ces poudres, mais seulement au moment où le commencement de la moisissure était visible à l'oeil nu. En même temps, comme contrôle, on déterminait l'humidité de la manière ordinaire.

Résultats des recherches.

I. Expériences à 100% d'humidité relative.

Dans les expériences précédentes nous avons trouvé qu'à une humidité relative de 100% la poudre moisissait au bout de deux mois. C'est pourquoi il nous a semblé d'abord suffisant de déterminer la teneur en humidité et le nombre de germes une fois par semaine. Au bout d'une semaine nous avons déja pu constater dans une poudre un accroissement de moisissure tandis qu'au bout de 14 jours la plupart des poudres étaient complètement moisies. Le tableau suivant nous donne le résultat des déterminations de la teneur en humidité et du nombre de germes.

Tableau IV.

100% d'humidité relative	A		B		C		D		Poudre Krause de l'expérience précédente	
	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes
Etat du début	2.7	53000	2.4	5900	4.0	9300	3.1	478000	5.9	12200
Après 7 jours	23.6	466000	21.8	3600	20.3	2200	15.3	68000	13.7	1500
Après 14 jours	26.8	21000 [1]	23.0	6400	21.1	56000	21.6	135000	14.9	2900
Après 21 jours		[2]	26.9	35000 [1]	24.9	[2]	24.9	[2]	17.3	181000
Après 25 jours	—	—	—	—	—	—	—	—	20.0	[1]

[1]) commencement de moisissure. [2]) moisie complètement.

Tableau IV (suite).

100% d'humidité relative	E		F		G		H	
	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes
Etat du début	2.6	29000	6.0	24000	2.9	31500	4.1	33700
Après 7 jours	21.4	18800	23.5	170000	23.5	56000 [1])	21.5	271000
Après 14 jours	24.9	66000 [1])	25.2	124000 [1])	24.9	[2])	28.5	[2])

[1]) commencement de moisissure. [2]) couverte de moisissure.

Par ces données on ne peut pas voir le cours du nombre de germes dans la conservation à 100% d'humidité relative. Il y a un indice que le nombre de germes diminue d'abord, ici également. Dans certains cas cependant on pouvait constater une augmentation au bout d'une semaine déjà, ce qui indiquait un commencement de moisissure.

Le moment où la moisissure se produit n'a pas été constaté exactement. Selon ces déterminations nous ne trouvons pas de

grandes différences dans les valeurs concernant la teneur en humidité de la poudre qui vient de moisir. Les valeurs vont de 26.8 à 23.5 pour les nouveaux échantillons examinés; la poudre Krause qui a été employée dans l'expérience précédente faisait seule exception. Le temps qui s'écoule avant que la moisissure se produise était, ici encore, plus long, ce qui permettait de constater plus exactement le moment où la moisissure se produisait. Il est possible que l'absorption d'humidité plus lente vienne de ce que la poudre était plus vieille.

L'expérience a été reprise. Cette fois-ci on a effectué les déterminations tous les quatre jours. On a restreint le nombre de poudres examinées. Le tableau V nous en donne le résultat.

Tableau V.

100% d'humidité relative	A		B		C	
	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes
Etat du début	3.2	17500	3.2	5700	4.0	3700
Après 3 jours	16.5	8000	18.9	1900	14.2	1900
Après 6 jours	15.5	7000	19.4	2600	14.0	1300
Après 9 jours	19.9	8000	21.3	2200	20.1	700
Après 13 jours	20.9	7200	20.8	3200	17.8	500
Après 18 jours	—	—	30.8	[2])	—	—
Après 19 jours	—	—	—	—	24.8	[1])
Après 21 jours	28.8	[2])	—	—	—	—

[1]) commencement de moisissure. [2]) moisie complètement.

Tableau V (suite).

100% d'humidité relative	D		G		F	
	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes
Etat du début	3.6	290000	3.8	22500	6.1	15400
Après 3 jours	19.6	63000	14.8	8100	21.6	9000
Après 6 jours	14.7	66300	13.8	1800	16.3	1500
Après 9 jours	21.8	58200	20.3	16500	20.9	4700
Après 13 jours	22.7	22100	18.2	10800	23.2	1500
Après 18 jours	33.5	[1])	—	—	—	—
Après 19 jours	—	—	18.8	[1])	—	—
Après 21 jours	—	—	—	—	33.4	[1])

[1]) moisie complètement.

Par les données de ce tableau on ne peut pas déterminer exactement la teneur en humidité où la poudre moisit. Le degré d'humidité des différentes poudres où l'on a constaté la moisissure dif-

férait et allait de 18.8 à 33.5%. La valeur de 18.4% trouvée dans l'expérience précédente est donc probablement dans les circonstances données une valeur minimum.

II. Expériences à 90% d'humidité relative.

Ces expériences ont été faites tout à fait de la même manière. Seulement, ici comme dans l'expérience à 85% d'humidité, il a été possible d'examiner la poudre une fois par semaine. L'absorption d'humidité avait lieu ici beaucoup plus graduellement que dans une atmosphère plus humide.

Tableau VI.

90% d'humidité relative	A		B		C		D	
	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes
Etat du début	3.2	46400	4.7	4800	7.9	1800	4.0	270000
Après 8 jours	20.2	15200	23.2	23400	20.2	3800	22.4	18400
Après 14 jours	21.4	7100	24.8	3300 [2]	21.0	2000 [2]	20.4	20000 [1]
Après 21 jours	26.8	8500 [1]	—	—	—	—	—	—

[1]) commencement de moisissure. [2]) moisie.

Les résultats du tableau VI nous montrent qu'il est probable que la teneur en humidité où la moisissure se produit à 90% d'humidité relative est plus basse qu'à 100%. Les valeurs obtenues pour cette teneur sont passablement plus élevées que les valeurs trouvées dans l'expérience précédente (voir tableau I).

III. Expériences à 85% d'humidité relative.

Les résultats de ces expériences sont réunis dans le tableau VII.

Tableau VII.

85% d'humidité relative	A		B		C		D	
	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes	humidité %	nombre de germes
Etat du début	3.2	46400	4.7	4800	7.9	1800	4.0	270000
Après 8 jours	14.6	8200	14.6	3400	11.6	1200	14.4	54000
Après 15 jours	15.5	7900	15.7	3800	13.3	1200	13.3	7000
Après 22 jours	16.3	6400	15.4	3300	13.2	600	15.4	19600
Après 27 jours	16.6	3700 [1]	16.0	5200 [1]	—	—	15.9	9800 [1]
Après 36 jours	—	—	—	—	14.3	1800	—	—
Après 38 jours	—	—	—	—	14.4	3700 [1]	—	—

[1]) commencement de moisissure.

De ces données nous pouvons conclure, que la teneur en humidité où se produit la moisissure est environ de 16%. Selon la méthode décrite précédemment, avec une couche épaisse de poudre, nous avons trouvé une valeur plus basse (voir tableau III).

Au sujet de la marche du nombre de germes nous pouvons remarquer qu'ici, de nouveau, une baisse a d'abord été constatée.

IV. Expériences à 75% d'humidité relative.

Comme nous l'avons exposé ci-dessus, ces expériences ont été

Tableau VIII.

75% d'humidité relative	A		B		C		D		E	
Etat du début	3.0	3.0	2.4	2.4	3.5	3.5	3.1	3.1	2.6	2.6
Après 4 jours	13.2	12.8	21.6	20.2	12.5	12.4	11.8	11.9	18.6	19.1
Après 8 jours	12.9	12.7	18.4	16.8	12.5	12.4	11.8	12.2	16.6	17.1
Après 11 jours	12.9	12.4	16.5	15.3	12.4	12.4	11.7	12.5	14.9	15.0
Après 17 jours	13.3	12.7	13.6	12.7	12.2	12.9	12.2	12.6	13.1	13.3
Après 20 jours	13.1	12.8	13.5	12.8	12.4	12.6	11.8	12.5	12.5	12.4
Après 24 jours	13.2	13.9	13.6	12.9	12.6	12.6	11.9	12.6	13.0	12.9
Après 28 jours	13.1	12.7	13.7	12.9	12.6	12.4	12.0	12.7	12.7	12.3
Après 34 jours	13.2	13.0	13.3	12.9	12.7	12.6	11.7	13.1	12.7	12.5
Après 50 jours	12.6	12.7	13.2	12.3	12.1	12.4	11.7	12.2	12.0	12.3
Après 56 jours	12.6	11.8	—	—	11.1	10.9	—	—	—	—
Après 57 jours	12.6	12.3	—	—	12.0	11.7	—	—	—	—
Après 58 jours	—	—	—	—	—	11.7	—	—	—	—
Après 62 jours	12.9	12.0	—	—	12.2	—	—	—	—	—
Après 65 jours	13.0	—	—	—	—	—	—	—	—	—
Après 68 jours	—	—	—	—	—	—	—	—	—	—

75% d'humidité relative	F		G		H		Poudre Krause expérience précédente	
Etat du début	6.0	6.0	2.9	2.9	4.1	4.1	5.9	5.9
Après 4 jours	14.8	14.9	12.1	12.0	20.5	21.1	12.8	13.6
Après 8 jours	15.9	15.1	14.2	12.0	18.0	18.2	12.5	14.1
Après 11 jours	16.4	14.8	13.5	12.2	15.6	15.7	12.8	14.3
Après 17 jours	16.6	14.9	13.6	12.3	12.4	12.8	12.8	13.9
Après 20 jours	16.4	14.6	13.4	11.8	—	—	12.8	13.9
Après 24 jours	16.4	14.4	13.6	12.2	—	—	13.1	13.8
Après 28 jours	14.6	13.7	13.7	12.1	—	—	13.0	13.2
Après 34 jours	15.3	14.8	14.3	12.6	—	—	12.9	13.2
Après 50 jours	15.3	14.0	13.6	12.2	—	—	12.8	13.5
Après 56 jours	12.0	12.4	13.6	11.8	—	—	13.0	13.3
Après 57 jours	14.2	13.9	13.3	12.4	—	—	12.4	12.9
Après 58 jours	—	—	—	—	—	—	12.2	12.8
Après 62 jours	14.2	—	13.5	12.2	—	—	—	—
Après 65 jours	—	—	13.7	—	—	—	—	—
Après 68 jours	—	—	13.2	—	—	—	—	—

faites pour déterminer la teneur en humidité dans l'état d'équilibre. Cette teneur a été calculée au moyen de la hausse de poids mesurée. Les résultats obtenus sont donnés en double dans le tableau VIII. Dans la fig. 5 nous donnons comme exemples caractéristiques, la marche de la teneur en humidité dans les poudres A et B.

Les valeurs indiquées dans le tableau sont celles qu'on a constatées jusqu'au début de la moisissure; dans le graphique on trouve la marche de la teneur en humidité pendant 50 jours.

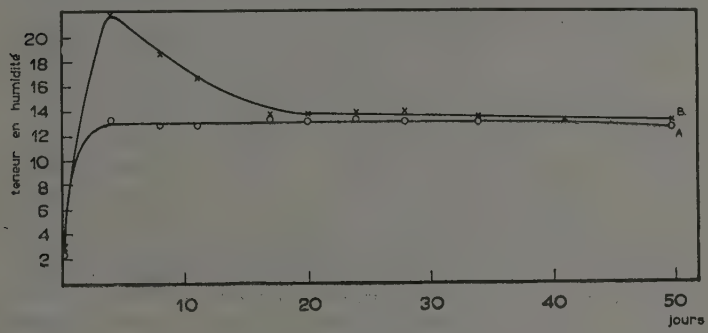

Fig. 5. Cours de la teneur en humidité pour les poudres A et B conservées à l'air contenant 75% d'humidité relative.

Dans le tableau IX nous donnons l'état final des poudres. A côté de la teneur en humidité, calculée d'après la hausse de poids trouvée, nous donnons les valeurs pour la teneur que nous avons trouvée par une détermination directe en séchant la poudre conservée jusqu'au poids constant à 103° C.

Si nous portons d'abord notre attention sur les résultats obtenus en ce qui concerne la teneur en humidité des poudres en état d'équilibre, alors nous remarquons en premier lieu qu'en effet un tel équilibre est atteint. Les valeurs obtenues pour cette teneur en humidité dans les différentes poudres varient de 14.2 à 11.7%. Nous n'avons donc pas trouvé de grandes différences.

Nous avons bien trouvé une différence dans la manière de laquelle cet état d'équilibre de la teneur en humidité a été atteint. Les poudres B, E et H avaient un caractère à part. Tandis que les autres poudres avaient déjà atteint leur maximum d'humidité au bout de 4 jours et qu'en les conservant plus longtemps elles restaient assez constantes, ces poudres (B, E et H) montraient une assez grande hausse pendant les 4 premiers jours et rebaissaient fort ensuite en atteignant une valeur conforme à celle des autres poudres. Supplee et Tamsma ont également constaté ce sommet dans la courbe, dans la conservation à 50%, ou plus, d'humidité relative.

Naturellement, il est possible que les autres poudres aient marqué un tel sommet, mais en tout cas elles étaient déjà redes-

Tableau IX.

75% d'humidité relative	durée de conservation (en jours)	teneur d'humidité		nombre de germes	
		calculée	déterminée	bactéries	moisissures
A	65	13.0	10.1	9000	300
	62	12.0	—	5600	1300
B	50	13.2	11.7	1700	3200
	50	12.3	12.1	1400	6900
C	65	12.2	9.9	600	8400
	58	11.7	11.0	1100	7100
D	50	11.7	10.5	1900	0
	50	12.2	10.2	1200	innombrables
E	50	12.0	9.8	1700	200
	50	12.3	—	1600	0
F	62	14.2	10.9	2500	3800
	57	13.9	10.2	2300	3800
G	68	13.2	11.3	1100	200
	62	12.2	12.2	300	400
H	17	12.4	—	—	—
	17	12.8	14.5	—	—
Poudre Krause de l'expérience précédente	58	12.2	10.8	1200	5000
	58	12.8	11.2	1700	300

cendues au point d'équilibre de la teneur en humidité au bout de 4 jours. Cette possibilité est même très probable, vu les constatations de SUPPLEE, qui a trouvé le maximum à 50% après 33 heures.

Enfin, quelques mots sur le début de la moisissure à 75% d'humidité relative.

La poudre H marquait à cet égard le plus grand écart. Elle était déjà moisie complètement après 17 jours tandis que pour les autres poudres, le temps qui s'écoulait avant que la moisissure fût constatable, variait de 50 à 68 jours.

Par cette expérience nous ne voyons pas d'une manière évidente pourquoi, dans ces cas, la moisissure ne se produit que si tard. Comme nous l'avons déjà dit, dans la plupart des cas la teneur en humidité définitive est déjà atteinte au bout de 4 jours. Le dit phénomène sera probablement en rapport avec le fait que la plupart des moisissures à 75% d'humidité relative ne se développent pas, ou seulement très lentement. Cette poudre, moisissant plus vite, aurait alors contenu des spores d'une sorte particulièrement xerophile. La teneur en humidité où se produit la moisissure à

75% d'humidité relative, se trouve encore beaucoup plus basse que la teneur en humidité, correspondant à une humidité relative plus élevée et se rapproche plus de la „teneur en humidité critique" dont nous avons parlé au commencement du paragraphe 4.

Il faut attirer l'attention sur le fait qu'il y a une assez grande différence entre la teneur en humidité calculée d'après la hausse de poids et la teneur déterminée de la manière ordinaire. En laissant la poudre H à part [1]), alors, nous voyons que la teneur en humidité déterminée de la manière directe est toujours plus basse que celle qui a été calculée. Cela pourrait indiquer qu'une partie de l'eau absorbée est si intimement liée qu'elle ne se dégage plus lorsqu'elle est chauffée à 103° C.

La détermination du nombre de germes montre qu'à la suite d'une si longue conservation le nombre de bactéries est à peu près égal dans toutes les poudres. Ce nombre de bactéries est très réduit. Le nombre de moisissures trouvées est assez arbitraire. Une différence importante entre les expériences précédentes faites dans une humidité relative plus élevée et cette dernière c'est que dans celle-ci l'équilibre de la teneur en humidité s'atteint en premier lieu et que ce n'est qu'après que la moisissure se produit. Par ces circonstances il est plus facile de déterminer au juste la teneur en humidité au commencement de la moisissure. Dans les teneurs en humidité relative plus élevées il semble qu'au début de la moisissure l'équilibre dans l'état d'humidité de la poudre n'est pas encore atteint. Ici la poudre moisit déjà lorsqu'on peut s'attendre à une hausse importante dans la teneur en humidité. Le développement de la moisissure empêche ici la détermination de la teneur en humidité dans l'état d'équilibre.

4. Relation entre la teneur en humidité où se produit la moisissure et la tension de vapeur relative de l'ambiance.

Dans les expériences de conservation en couches minces nous avons constaté que la teneur en humidité où se produit la moisissure dépend aussi de l'humidité relative du milieu où la poudre est conservée. Suivant le tableau X, où sont réunies ces teneurs en humidité, ce degré d'humidité est d'autant plus haut que l'humidité relative est plus élevée.

Il est admissible que cette dépendance difficile à comprendre, à première vue, n'est qu'apparente. En réalité la moisissure pourrait dans tous les cas commencer à une même „teneur en humidité critique". Comme il s'écoule pourtant assez de temps avant que la moisissure en soit au critérium „moisissure visible", il faudra tenir compte que pendant cette période le procès d'absorption d'humidité continue et devient ensuite d'autant plus rapide, par la force des choses, que l'humidité relative du milieu est plus

[1]) Cette poudre était toute moisie lorsque la teneur en humidité était déterminée. Les autres poudres commençaient à moisir lorsqu'on déterminait leur teneur finale.

grande. Les différences dans le développement de la moisissure dans les différentes humidités relatives ne seront pas assez grandes pour compenser les différences dans la vitesse de l'absorption de l'humidité.

Tableau X.

humidité relative	100%	90%	85%	75%
A	26	25	17	12
B	27	20	16	12
C	25	20	14	13
D	25	22	16	12
E	25	—	—	12
F	25	—	—	14
G	24	—	—	13
H	25	—	—	12
Poudre Krause de l'expérience précédente	20	—	—	12

Résumé.

1. Les expériences ont été faites dans le but de suivre le cours du nombre de germes et de la teneur en humidité du lait sec en le conservant à différents degrés d'humidité relative. On a commencé par conserver la poudre en couche d'une épaisseur quelconque. Plus tard on a conservé des échantillons de fabrication différente en couches minces.
2. Si on met le lait sec dans un milieu dont il peut absorber l'humidité alors une forte baisse du nombre total de germes a d'abord lieu. Puis ce nombre de germes reste assez constant pendant longtemps pour remonter de nouveau quand l'humidité dans la poudre a atteint un certain niveau. Au-dessous de cette teneur en humidité critique le développement d'une flore microbienne considérable n'est pas possible.
3. La teneur en humidité critique est indépendante de la tension de vapeur de l'air où la poudre a absorbé de l'eau.
4. La teneur en humidité où la moisissure visible se produit est d'autant plus basse que la poudre est conservée dans une humidité relative plus basse.
5. Dans une humidité relative de 100%, 90% et de 85% la moisissure se produit avant que la teneur en humidité de la poudre soit en équilibre avec l'humidité de l'air ambiant; à 75% d'humidité relative l'équilibre s'est déjà établi avant le début de la moisissure.
6. Avant d'atteindre l'équilibre à 75% d'humidité relative quelques poudres ne reprenaient de l'humidité que beaucoup plus tard, jusqu'à une teneur de 20% environ, puis cette teneur rebaissait

et atteignait une valeur normale conforme à celle des autres poudres qui absorbaient l'eau immédiatement jusqu'à cette teneur normale.

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K. C. Winkler, Iets over de stikstof-stofwisseling bij *Bact. coli* (Aspects of the nitrogen metabolism of *B. coli*). Chem. Weekblad **40**, 147, 1943.

The quotient of the decrease of ammonium in the medium and the number of bacteria present is determined after various intervals in a medium containing ammonium sulphate as sole source of nitrogen on which *B. coli* was cultivated. Along with the increase in age of the cultures the quotient decreases considerably (1000× or more).

As it is impossible that the bacteria would have contained initially a 1000 fold amount of nitrogen, it is to be accepted that at the start a part of the ammonium is changed into amino-nitrogen which is not taken up by the bacteria. This may lead to the supposition that the synthesis of amino acids is an exogenic process located in the membrane of bacteria. Amino acids are probably produced already in the lag phase; perhaps a definite external concentration has to be reached before the synthesis of protein may occur. W. H.

N. Savrij, Eenige vergelijkende onderzoekingen over het aantoonen van coli-bacteriën in gepasteuriseerde melk (Some comparitive investigations of the means of detecting *B. coli* in pasteurised milk). Chem. Weekblad **38**, 114, 1941.

Compared are: The method of the milk decree 1916 (enrichment in acid broth), the method of an official control instance (enrichment in meat broth with lactose and neutral red and cultures on Endo plates), and the method of the laboratory of the Cooperative Plant of Milk products at Bedum (enrichment in a peptone-lactose-brillant green-gall medium and culture on eosine-methylene blue plates).

The results vary, especially when the fermentation test is negative. It became evident that on the Endo plate red colonies even those with a metallic lustre are often not due to *B. coli*. On the other hand on the eosine-methylene blue plates colonies with a metallic lustre are always formed by bacteria of the coli group.

Therefore it seems desirable to base the research for *B. coli* in pasteurised milk on: 1. the occurrence of gas in a lactose containing medium. 2. the metallic lustre of colonies on eosine-methylene blue plates. W. H.

A. J. Kluyver, De microbiologische grondslagen der voedselconserveering (The microbiological basis of the conservation of food). Chem. Weekblad **38**, 383, 1941.

The paper contains chiefly a discussion of the action of high and low temperatures and more especially the harmful action of high temperatures on bacteria. The loss of the reproductional power is

taken as a criterium for the death of the bacteria. By means of graphs the letality of the bacteria and the spores as a function of time at various temperatures is visualised. Moreover of importance are: the original number of microbes, the state of maturity of the spores present, the chemical composition and especially the pH of the surrounding medium. Some results arrived at under practical conditions are discussed.

Besides this the advantages or disadvantages of temperatures beneath and just above the freezing point are discussed. Here as well the initial number of microbes is of importance and also the degree of moistness.

The influence of carbon dioxide, ozonising, drying and conservation by means of salt, sugar and acids is shortly discussed.

W. H.

L. BIJLMER, Aetiologie der Influenza, De isoleering van het influenza-virus tijdens de epidemie van 1941 te Groningen (Etiology of Influenza. The isolation of the influenza virus during the epidemic of 1941 at Groningen). Diss. Groningen, 1943. H. E. Stenfert Kroese's Uitgevers-Mij N.V., Leiden.

The experimental studies on influenza, during the epidemic of January-February 1941 at Groningen (Holland), were carried out according to the technique evolved by WILSON SMITH, ANDREWES and LAIDLAW, of the National Institute for Medical Research at Hampstead (London). The investigations were done in a laboratory specially built in 1940 for the study of experimental influenza in ferrets and since 1941 incorporated in the Institute for Hygiene and Bacteriology of the State University.

During the period of January 9th to February 7th, material from 13 patients, partly throat-washings, partly suspensions of sputa, obtained during the first days of illness, was inoculated in ferrets, which were kept under rigid measures of isolation. The temperature was read twice daily and the clinical signs were recorded.

In 5 cases the ferrets developed a typical influenza infection, with the characteristic fever peak on the second or third day and catarrhal signs of the nose, sometimes of the conjunctiva. In all these cases influenza antibodies against the virus strain WS could be demonstrated in the ferret convalescent serum by means of the mouse protection test.

In two other cases the signs in the ferrets were doubtful, but the ferret convalescent serum contained antibodies against the influenza virus, so that these animals must be assumed to have gone through a subclinical infection.

In 4 of the 6 remaining cases in which the ferrets did not show any reaction, the patients' convalescent serum neither showed any rise in antibodies. In the remaining 2 both the mouse protection and complement fixation test of the patients' sera gave positive results, so that missed ferret inoculation must be assumed.

Three virus strains were isolated, from three of the enumerated cases. After some ferret passages (3, 4 and 5) the virus was adapted to mice. The strains produced lethal pulmonary lesions in the mice after 9, 3 and 7 passages respectively.

In the course of the ferret and mouse passages the virus suspension was filtered through a collodium membrane (average pore diameter 0.6 μ). The throat-washings and sputa from the patients were not filtered before being inoculated in the ferrets, in order not to reduce the virulence of the pathogenic agent.

The nature of the pulmonary lesions of the mice was checked microscopically by sectioning the lung. Hereby we accepted the criteria described by STRAUB.

The analysis of the three isolated influenza virus strains did not show any mutual antigenic differences. The new strains were not found to be identical with any of the English „specific" strains.

The immunological study of the sera from fifty influenza patients demonstrated, that in 33 cases the influenza virus had been the etiological agent; among these were 13 cases of pneumonia, following an attack of influenza.

Prophylaxy and therapy by means of vaccine and immune serum are discussed. A multivalent anti-influenza serum was prepared by hyperimmunising rabbits with various virus strains. Some prophylactic effect of rabbit immune serum, intranasally administered to mice infected with influenza virus, could be noted.

The aim of this study may not be considered reached by the ascertainment of the etiology in a single influenza epidemic. This work must be seen as a preparation for the study of an influenza pandemic. For the Netherlands the research work in this field is being organised by the Institute for Preventive Medicine (Instituut voor Preventieve Geneeskunde) at Leiden.

L. BIJLMER.

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